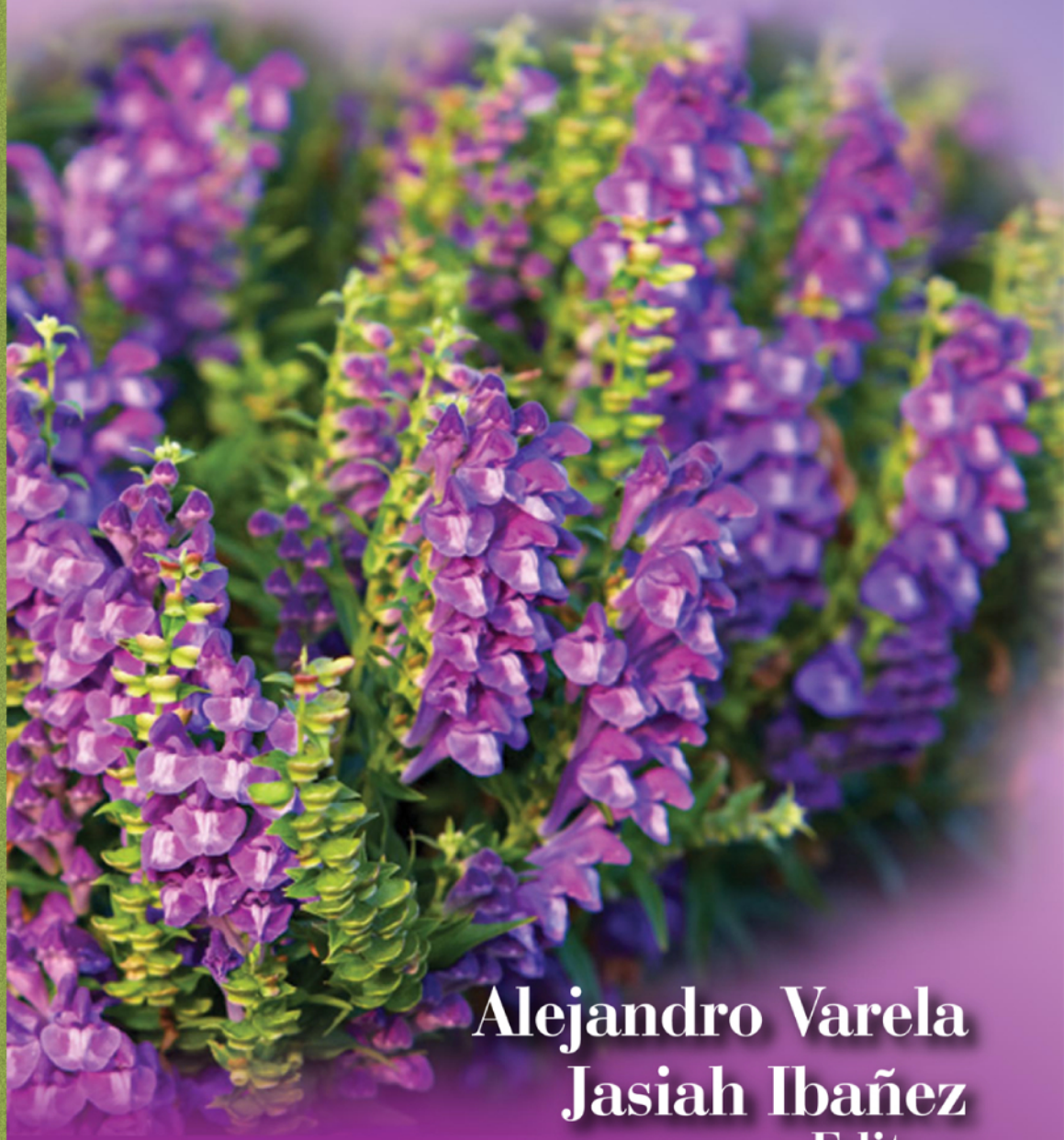
Biotechnology in Agriculture, Industry and Medicine Series

Medicinal Plants

Classification, Biosynthesis and Pharmacology



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BIOTECHNOLOGY IN AGRICULTURE, INDUSTRY AND MEDICINE SERIES

MEDICINAL PLANTS: CLASSIFICATION, BIOSYNTHESIS AND PHARMACOLOGY

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MEDICINAL PLANTS: CLASSIFICATION, BIOSYNTHESIS AND PHARMACOLOGY

ALEJANDRO VARELA
AND JASIAH IBAÑEZ
EDITORS

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Preface

Plants have been the main source of medicines since ancient times. Practically all human societies have utilized plants not only as sources of nutrition but also as therapy against diseases and ailments. Considering the fact that the synthesis of a pharmaceutical requires an enormous investment of research and money, the discovery of useful medicinal plants which have been used for millennia is very appealing. This book examines Scutellaria baicalensis, one of the most widely used medicinal plants whose roots have been used for anti-inflammation, anticancer, decreasing blood pressures, reducing the total cholesterol level and treating bacterial and viral infections. The pharmacological, toxicological reports and clinical applications of B-carotene, an organic compound abundant in plants and fruits, is also explored. Furthermore, diabetes is a metabolic syndrome resulting from low levels of insulin. This book focuses on recent examples of traditional medicines and foods that have been validated by scientific evaluation as having promising activity for the prevention and/or treatment of diabetes. Other chapters in this book describe compounds found in some plants that have been tested in different bioassays and showed anti-mycobacterial activity, the advantages of the novel quality control near-infrared spectroscopy (NIRS) tool in medicinal plant analysis, and a quantitative analysis of polysaccharides from medicinal plants and fungi.

Chapter 1 - Several carotenoids show enhancement of the immune response, inhibition of mutagenesis, reduction of induced nuclear damage, and protection from various neoplastic events in cells, tissues, and whole animals. Carotenoids also protect against photo-induced tissue damage. Some carotenoids, including β-carotene, quench highly reactive singlet oxygen under certain conditions and can block free radical-mediated reactions. There is a growing body of literature on the effects of β-carotene in human chronic diseases, including cancer. Evidence from observational epidemiological studies has shown that a high consumption of fruits and vegetables rich in carotenoids is associated with a low risk for cancer. However, some human intervention trials failed to demonstrate prevention of cancer by β-carotene supplements. Several studies have indicated that among subjects who neither smoked cigarettes nor drank alcohol, β-carotene was associated with a marked decrease in the risk of one or more recurrent adenomas but β-carotene supplementation conferred a modest increase in the risk of recurrence among those who smoked. An increase in the risk of lung cancer among smokers and asbestos workers who took β-carotene supplements is also

reported. In fact this trial raises the possibility that these supplements may actually have harmful as well as beneficial effects. Alcohol intake and cigarette smoking appear to modify the effect of β-carotene supplementation on the risk of colorectal adenoma recurrence. Similarly, serum β-carotene levels have been associated with a decreased chance of developing cancer. This results show a remarkable consistency for the association of increased lung cancer risk with low amounts of dietary β-carotene or low plasma β-carotene concentrations. For stomach cancer, the evidence is also consistent, although the number of studies is more modest. For breast and prostate cancer, the studies indicate no consistent association of plasma or dietary β-carotene and reduced cancer risk. For colorectal cancer, the effect will be moderate, if existent. Whatever the results of these trials, carotenoids clearly show biological actions in animals distinct from their function as precursors of vitamin A. This review is an up-to-date and comprehensive analysis of pharmacological, toxicological reports and clinical applications of the β-carotene.

Chapter 2 - This study reviews the main native medicinal plants that compose the pharmacopoeia of the highland population in the province of Córdoba, central Argentina. From a methodological point of view, the authors combine first-hand information from previous investigations, field documents on medicinal species and their applications, and the results of other ethnobotanical studies on the region. The authors provide an extensive list of species and applications, a thorough description of the habits and therapeutic practices in which the species are used, and present the most characteristic features of peasant ethnomedicine. The authors also describe the main specific features of the etiological explanations given for diverse maladies and different forms of diagnosis and treatment. Based on the use of quantitative indicators such as the number of uses, the consensus and relative importance for a particular use and pharmaco-botanical information, the authors indicate the native species that would be interesting to apply in primary health care. Finally, the authors suggest practices regarding the conservation of these species taking into consideration their distribution, ecology and botanical status.

Chapter 3 - Gastric and duodenal ulcers affect a considerable amount of people in the world. Ulcer occurs when there is a disturbance of the normal equilibrium caused by either enhanced aggression or diminished mucosal resistance of gastrointestinal tract. Mucosa defends gastrointestinal tract of acid, pepsin, bile, leukocyte infiltration and external substances such as alcohol, caffeine, chilli or certain drugs such as NSAIDs. The defense mechanisms of the gastrointestinal mucosa mainly consist of functional, humoral and neuronal factors. Mucus alkaline secretion, mucosal microcirculation and motility act as functional factors, while prostaglandins and nitric oxide (NO) act as humoral factors, and capsaicin-sensitive sensory neurons (CPSN) act as neuronal factors. Several plants containing triterpenoids have been shown to possess anti-ulcer activity. The gastroprotective effects of triterpenoids have been studied on ethanol or NSAIDs-induced gastric injury models. These models induced impairment in the mucosal defense process with the consequent gastric damage. The principal mechanism of gastroprotection of triterpenoids has been reported by the activations of mucous membrane secretion instead of the inhibition of gastric acid secretion. Chemically, this gastroprotective effect has been referred to the presence of a hydroxyl group free or derivative at position C-3 for sterols and triterpenoids. Some pharmacological gastroprotective mechanisms for this kind of natural products has been

attributed to the role of prostaglandins, nitric oxide (NO), sulfhydryl groups (-SH), and capsaicin-sensitive afferent neurons. Besides, recently leukocyte adherence, TNF-α and hydrogen sulfide has been implicated on mucosal defense mechanism, however it is unknown on triterpenoids gastroprotection.

Chapter 4 - *Scutellaria baicalensis* Georgi is one of the most widely used medicinal plants, and is officially listed in the Chinese Pharmacopoeia. Its roots have been used for anti-inflammation, anticancer, decreasing blood pressures, reducing the total cholesterol level, treating bacterial and viral infections of the respiratory and the gastrointestinal tract, cleaning away heat, moistening aridity, purging fire and detoxifying toxicosis. This plant also possesses cholagogic, diuretic, and cathartic actions. Some concentrated composite herbal preparations containing *Scutellaria baicalensis* Georgi as a major ingredient in their prescriptions are widely used in oriental countries. *Scutellaria baicalensis* Georgi contains a variety of flavones, phenylethanoids, amino acids, sterols and essential oils. Its dried roots contain over 30 kinds of flavonoids, such as baicalin, baicalein, wogonin, wogonin 7-O-glucuronide and oroxylin A. The flavonoids are the main active components in *Scutellaria baicalensis* Georgi. This chapter provides up-to-date coverage of this class of flavonoids in regard to chemical structures, natural resources, biosyntheses, analytical methods and biological activities. Special attention is paid to both biosyntheses and biological activities including antioxidant and free radical scavenging, anti-inflammation, anticancer, antibacterium, anti-HIV, anti-hepatitis B virus, anti-respiratory syncytial virus and anti-SARS coronavirus properties. The structural diversity and the pronounced biological activities encountered in the flavonoids of *Scutellaria baicalensis* indicate that this class of compounds is worthy of further studies that may lead to new drug discovery. The review provides an account on our research work combined with a reference of the information obtained in both the English and Chinese literature.

Chapter 5 - Diabetes is a metabolic syndrome resulting from low levels of insulin. Common symptoms are hyperglycemia, polyuria, polydipsia, blurred vision, lethargy and weight loss. The increasing worldwide incidence of diabetes mellitus in adults constitutes a major global public health burden. The World Health Organization (WHO) estimates that currently more than 180 million people worldwide have diabetes. This number is likely to double by 2030 when it is predicted that India, China and the United States will have the largest number of people with diabetes.

Plants have been the main source of medicines since ancient times. Despite tremendous advances in medicinal chemistry, synthetic drugs have not provided cures to many diseases due to their adverse side effects or diminution in response after prolonged use. Plants are the richest source of natural compounds and continue to provide new chemical entities for the development of drugs against various diseases like cancer, diabetes, inflammation, hypertension and neurodegeneration. As such, there is renewed interest in traditional medicines with the belief that plant-derived drugs are generally less toxic and safer than synthetic drugs. With respect to diabetes, numerous studies have indicated that plant-derived chemicals may be useful in the therapeutic treatment of diabetes. However, before the development of therapeutic insulin, diet was (and still is) the main method of treatment and modern treatment focuses on a combination of drugs and diet. Dietary measures included the use of traditional medicines mainly derived from plants. While drugs will continue to be an

important part of diabetes therapy, the mass of evidence available in the literature regarding the medicinal properties of vegetables, fruits and other herbs, suggests that diet (including herbal medicines) should not be ignored or neglected.

This review will focus on recent examples of traditional medicines and foods that have been validated by scientific evaluation as having promising activity for the prevention and/or treatment of diabetes. Intriguing questions that await further elucidation include how plants, plant-derived molecules and diet can be used in the future to complement current treatment strategies for diabetes.

Chapter 6 - Tuberculosis is an infectious, primary pulmonary disease, caused by *Mycobacterium tuberculosis* that remains an important public health problem worldwide with approximately nine million new cases and two million deaths per year. TB is considered the most important disease caused by a single infectious agent and its control has been difficult due to the lack of an effective vaccine, association with HIV infection and the progressive development of resistance to anti-TB drugs.

Alternative anti-mycobacterial drugs are urgently needed; studies have shown that medicinal plants traditionally used to treat respiratory diseases are a potential source of new and efficient compounds to treat tuberculosis.

In this chapter we will describe some compounds, found in plants that have been tested in different bioassays and showed anti-mycobacterial activity.

Chapter 7 - "*Angelica keiskei* AK", a health food, originated from Japan (*Umbelliferae*, "Ashita-Ba" in Japanese), has been distributed islandwide and widely consumed by the general public in Taiwan during the past twenty-five years. This plant was recognized as natural aromatic and an important medicinal plant of traditional Chinese herbs. Presently, this herb is treated as a diuretic, analeptic, lactagogue and has been recommended, cultivated, and propagated by the Taiwan Agricultural Research Institute (TARI). AK was sampled from five main planted areas to ensure diversity in the summer and spring harvest seasons in central Taiwan. Epithermal and instrumental neutron activation analysis (ENAA and INAA) revealed the presence of nearly twenty metals in the roots, fresh leaves and stems of the plant, as well as in end-products such as tea bags and capsules of the Taiwanese health food product. This research employed ENAA to identify aluminum (Al), arsenic (As), bromide (Br), chloride (Cl), iodine (I), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), antimony (Sb), and samarium (Sm) and INAA to identify chromium (Cr), iron (Fe), lanthanum (La), rubidium (Rb), scandium (Sc), selenium (Se), vanadium (V) and zinc (Zn). Some of these elements are classified as either toxic or essential to humans. In the collected samples the elements exist in widely differing concentrations, ranging from 10^5 to 10^{-2} μg/g from different farms. Zinc concentrations in the tea bags are higher than those in the drinking teas, Mg, and I were the first elements to be detected. The elemental concentrations and maximum daily intake (MDI) of this herb are compared with those of *Angelica sinensis* (Danggui in Mandarin), *Ligusticum chuanxiony* (Chuanxiong in Mandarin) and *Panax ginseng* (Ginseng in Mandarin) as well as with the recommended daily dietary intake values for Taiwanese consumers, developed by the WHO. The prescription (12 g/day for adult, 6g/day for children), the MDI of As is below that recommended by WHO/FAO, and thus the average daily intake of Al, Fe and Sc in Taiwan is probably excessive. However, the MDI of Cr, Fe, Mn, and Zn among five farms and available in the markets are all below the levels

recommended by WHO/RDA. Finally, the MDI of Al, Br, Cl, K, La, Na, Rb, Sb, Sc, Sm and V correlate closely with the levels recommended by WHO/RDA.

Chapter 8 - The humankind needs to keep on exploring, in a rational manner, the chemical substances offered by living organisms, learning, copying and imitating the nature in its potential and structural diversity offered by laboratories of vegetal and animal analysis. Learning the chemical dynamism adopted by fauna and flora organisms will undoubtedly help the scientific progress of the nations. Besides, it will provide contribution for a better quality of life, protection and survival, comprehension and conservation of environmental conditions on planet Earth (Turolla, Nascimento, 2006; David, David, 2006).

Chapter 9 - Echinodorus grandiflorus (Cham. & Schltdl.) Micheli and Echinodorus macrophyllus (Kunth) Micheli, are monocotyledonous species belonging to Alismataceae family. These plants are aquatic or semi-aquatic herbs, with submersed, floating or emersed leaves and with inflorescences that remain flourished during nearly 30 days. In Brazil, they are popularly known as "chapéu de couro" and have been used in the folk medicine in the treatment of several disorders. Its leaves are resources for very common teas, used as diuretic and anti-inflammatory, blood depurative, against arthritis and skin diseases, liver maladies and renal affections, as well as against amygdalitis, pharyngitis, stomatitis and gingivitis. Several researches have suggested promising results on medicinal activities of “chapéu de couro”. Some of those activities were observed in vivo, such as diuretic, anti-inflammatory, hypotensive and antihypertensive, antimicrobial, decholesterolizing, immunosuppressive and vasodilator. In vitro activities were also confirmed, such as trypanocidal, leishmanicidal and antineoplastic. In this work it is presented the ethno and experimental pharmacology, regarding the researches accomplished so far, besides the botanical characterization, geographic distribution, macro and microscopic description, chemical constituents and toxicology.

Chapter 10 - Near-Infrared spectroscopy (NIRS; 800-2500 nm) is a non-invasive spectroscopic tool enabling a fast qualitative and quantitative characterization of medicinal plants and their constituents down to the ppm-level. Treatment of spectra recorded with chemometrical and multivariate approaches allows determining chemical (e.g. secondary plant metabolites, leading compounds) and physical parameters (e.g. water, alcohol content) simultaneously by one single measurement lasting only a few seconds.

Liquid plant extracts are investigated in the transflection mode at thermostated conditions using light-fibre optics, dried parts of plant (flowers, leaves, roots) also in the reflection mode using a sample desk. For the quantitative analysis of secondary metabolites including 3′,4′,5′-trimethoxyflavone in *Flos Primulae veris*, hypericin and hyperforin in *St. John´s Wort*, etheric oils in *Achillea* species, a reference method based on liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE) is applied. Qualitative cluster analysis not only allows identifying different parts of a plant but also enables to distinguish different species, which is essential also in traditional Chinese medicine (TCM).

In the present contribution the main advantages of the novel quality control NIRS tool in medicinal plant analysis are pointed out and discussed in detail by several applications.

Chapter 11 - Practically all human societies have utilized plants not only as sources of nutrition but also as therapy against diseases and ailments. Considering the fact that the synthesis of a pharmaceutical requires an enormous investment of research and money, the

discovery of useful medicinal plants which have been used for millennia is very appealing. About 25% of all synthesized drugs are derived directly or indirectly from plants [1].

In the USA the market in plants used for medicinal purposes involved $4.8 billion in 2007 and $5 billion in Europe in 2003 (2-3). The increase in the demand for phytotherapeutic products in the USA has resulted in new rules starting in 2008 so that products adhere to Good Manufacturing Practices (2).

The European Union in 2004 passed a law permitting a simplified registration procedure for herbal medicines which have been used for at least 30 years (and 15 years in Europe). These phytotherapeutic products must have adequate documentation of nontoxicity with specific conditions of use (3).

In the 1990s the World Health Organization (WHO) stated that the use of traditional medicines was the only sustainable way to provide primary healthcare to individuals in developing nations [4]. An international meeting of 134 nations at Alma Ata in 1978 established the objective of providing adequate healthcare for all people in the world by the year 2000. In that year, non-governmental organizations from 92 nations met in Savar, Bangladesh to reaffirm the same goal.

Given this global situation, the patenting of plant medicines by pharmaceutical industries can constitute a problem. A pharmaceutical industry would be reluctant to engage in the high economic investment needed to carry out chemical, pharmacological and clinical studies without an economic endpoint or profit. The only reason that a pharmaceutical industry would be interested in investing in research in the area of herbal medicines would be a public refundability.

Chapter 12 - Polysaccharides isolated from medicinal plants and fungi exhibit multiple pharmacological activities, including anti-tumor, anti-oxidation, hypoglycemic activity and immune potentiation and so on. The biological activities of polysaccharides depend on their chemical characteristics. However, quality control of polysaccahrides is a challenge because of their complicate structure, macro-molecular mass, more characters showed relationship with the bioactivities and more potential symbols could be used as the evaluation indicators. In this review, qualitative assay including the tests of purity, molecular weight and its distribution, constituent monosaccharide composition and the ratio, the features of glycosidic linkages, as well as quantitative analysis of polysaccharides from medicinal plants and fungi were reviewed and discussed. Among the various means for quality control of polysaccharides, chromatographic and electromigratic methods including high performance liquid chromatography (HPLC) such as high performance anion exchange chromatography (HPAEC), size exclusion chromatography (SEC) and electrophoresis (e.g. capillary electrophoresis and gel electrophoresis) are powerful techniques. The perspective for quality control of polysaccharides has also been described.

Chapter 13 - Bacterial antibiotic resistance has become a serious problem of public health that concerns almost all antibacterial agents and that manifests in all fields of their application. Consequently, there is an increasing interest in the search for new compounds which can act by a direct antimicrobial effect or by inhibiting resistance mechanisms of microorganisms of medical importance. Medicinal plants nowadays remain a valuable source for this kind of compounds. The direct antimicrobial properties of a number of natural compounds have indeed been reported; such compounds act by many mechanisms, including:

(i) complexation with macromolecules such as proteins and polysaccharides, thus inhibiting their functions (polyphenols); *(ii)* disruption of microbial membranes (lipophilic flavonoids, terpenoids, plant defensins); and *(iii)* inhibition of adhesion of microbial proteins to host polysaccharide receptors (polypeptides). Medicinal plants also provide compounds which are not necessarily effective against microorganisms, but which enhance or restore the activity of antibiotics by inhibiting resistance mechanisms. These compounds belong to several phytochemical groups and act as inhibitors of efflux pumps (flavonoids, terpenoids, alkaloids); inhibitors of PBP 2a (quinones, terpenoids), enhancers of the permeability of bacterial membrane (terpenoids) and beta-lactamases inhibitors (alkyls gallates).

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Chapter 1

Biological Effects of β-Carotene

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Abstract

Several carotenoids show enhancement of the immune response, inhibition of mutagenesis, reduction of induced nuclear damage, and protection from various neoplastic events in cells, tissues, and whole animals. Carotenoids also protect against photo-induced tissue damage. Some carotenoids, including β-carotene, quench highly reactive singlet oxygen under certain conditions and can block free radical-mediated reactions. There is a growing body of literature on the effects of β-carotene in human chronic diseases, including cancer. Evidence from observational epidemiological studies has shown that a high consumption of fruits and vegetables rich in carotenoids is associated with a low risk for cancer. However, some human intervention trials failed to demonstrate prevention of cancer by β-carotene supplements. Several studies have indicated that among subjects who neither smoked cigarettes nor drank alcohol, β-carotene was associated with a marked decrease in the risk of one or more recurrent adenomas but β-carotene supplementation conferred a modest increase in the risk of recurrence among those who smoked. An increase in the risk of lung cancer among smokers and asbestos workers who took β-carotene supplements is also reported. In fact this trial raises the possibility that these supplements may actually have harmful as well as beneficial effects. Alcohol intake and cigarette smoking appear to modify the effect of β-carotene supplementation on the risk of colorectal adenoma recurrence. Similarly, serum β-carotene levels have been associated with a decreased chance of developing cancer. This results show a remarkable consistency for the association of increased lung cancer risk with low amounts of dietary β-carotene or low plasma β-carotene concentrations. For stomach cancer, the evidence is also consistent, although the number of studies is more modest. For breast and prostate cancer, the studies indicate no

consistent association of plasma or dietary β-carotene and reduced cancer risk. For colorectal cancer, the effect will be moderate, if existent. Whatever the results of these trials, carotenoids clearly show biological actions in animals distinct from their function as precursors of vitamin A. This review is an up-to-date and comprehensive analysis of pharmacological, toxicological reports and clinical applications of the β-carotene.

Introduction

Carotenoids are found almost everywhere in nature, but particularly among organisms that bask in the sun. These interesting compounds, most of which reveal a yellow to red color, have attracted the attention of biologists at least since the early 1800s. The colored compounds in plants, animals, and microorganisms were extracted and purified, and in time their structures were determined. Many treatises have been devoted to these compounds, the most comprehensive of which was edited by Isler in 1971.

Straub in 1971 listed the 273 compounds sufficiently characterized at that time to be clearly distinct from all others. An update of this key in 1987 expanded the list to 563 distinct compounds (Straub, 1987). Because *cis-trans* isomers of a given carotenoid are not listed separately, the actual number of naturally occurring carotenoids is significantly larger. Thus, carotenoids represent a very large group of substances with various structural characteristics and biological activities.

600 carotenoids from natural sources that have been characterized, fewer than 10% serve as precursor's of vitamin A. Many dietary carotenoids, both with and without provitamin A activity, are found in the blood and tissues of humans. β-Carotene, the most nutritionally active carotenoid, comprises 15-30% of total serum carotenoids (Bendich and Olson, 1989).

β-carotene is a member of the carotenoids, which are highly pigmented (red, orange, yellow), fat-soluble compounds naturally present in many fruits, grains, oils, and vegetables (green plants, carrots, sweet potatoes, squash, spinach, apricots, and green peppers). α, β, and γ carotene are considered provitamins because they can be converted to active vitamin A, which is a nutrient that is vital to growth and development. It is obtained in the diet from animal sources and is also derived from β-carotene in plant foods. It is broken down in the mucosa of the small intestine by β-carotene dioxygenase to retinal, a form of vitamin A and this is mainly stored in the liver in the form of esters of retinol. The β-carotene can also be absorbed and stored in the fatty tissue without being modified, producing a slightly yellow or orange color on the palms of the hands. Vitamin A and closely related molecules are also known as retinoids (Kennedy et al., 1996).

In human, absorbed β-carotene is converted into retinal in enterocytes and the liver by a specific enzyme (15,15′-dioxygenase), which generates retinal by central cleavage (Roos et al., 1998). Another metabolic pathway is eccentric cleavage of β-carotene via β-apocarotenals to retinal (Blomhoff et al., 1992). Retinal is then converted into retinol by dehydrogenases, and retinol is transported by retinol-binding protein, a specific plasma protein, to target tissues. Human epidermis contains two major retinoids (retinol and retinyl esters) and carotenoids (mainly β-carotene) (Vahlquist, 1982). Vitamin A can be stored in keratinocytes through esterification of retinol into retinyl esters. This step is catalyzed by two enzymes,

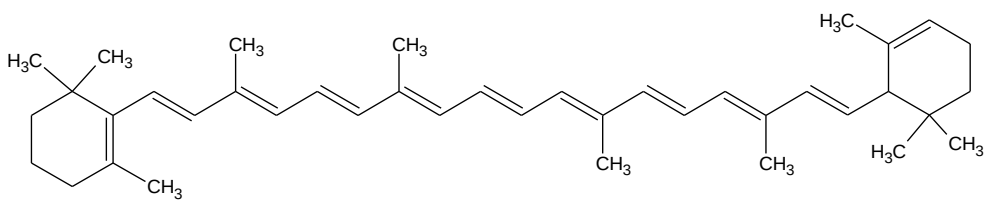
acyl-CoA: retinol acyltransferase (ARAT) and lecithin: retinol acyltransferase (LRAT); their expression is modulated by the differentiation state of the keratinocytes (Torma et al., 1988). Hydrolysis of retinyl esters into retinol is catalyzed by retinyl ester hydrolases. Retinol, via its oxidation into retinal, is a pro-hormone of retinoic acid (Siegenthaler et al., 1990), the biologically active form of vitamin A that modulates gene expression following its binding to nuclear receptors. Thus retinal, retinol, and its esters are endogenous precursors of the biologically active form of vitamin A (Antille et al., 2004)

Carotene is an orange photosynthetic pigment important for photosynthesis. It contributes to photosynthesis by transmitting the light energy it absorbs to chlorophyll. Chemically, carotene is a terpene, synthesized biochemically from eight isoprene units. β-carotene is composed of two retinyl groups.The two primary isomers of carotene, α-carotene and β-carotene, differ in the position of double bonds in the cyclic group at the end (*Pitchford, 2002*).

β-Carotene is the most abundant carotenoid in nature and most important for the human diet, so that gives its name to a whole group of biochemical compounds. Its structure was determined in 1930 by Paul Karrer work that earned him the Nobel prize in chemistry. This was the first time in history in which the structure of a vitamin or pro-vitamin was identified. The absorption spectrum of β-carotene shows two absorption peaks between 400 and 500 nm, corresponding to blue and green, so that the red-orange-yellow reflecting gives its characteristic color (Karrer, 1928).

The main properties of the β-carotene are:

- Antioxidant function: quenching the action of oxygen free radicals, inhibiting the peroxidation of lipids of the membranes.
- Protection from solar radiation (photoprotectors), encouraging the production of melanin and therefore a tan uniform and intense.
- Immune function: improved resistance to infection.
- Restoration and maintenance of the epithelial cells that are the cavities of the body (skin, glands, membranes, gastrointestinal mucosal).



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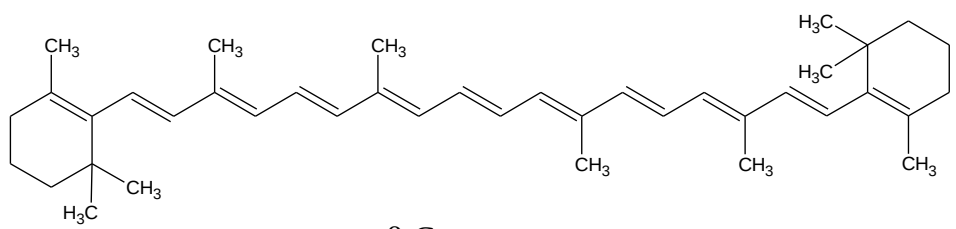

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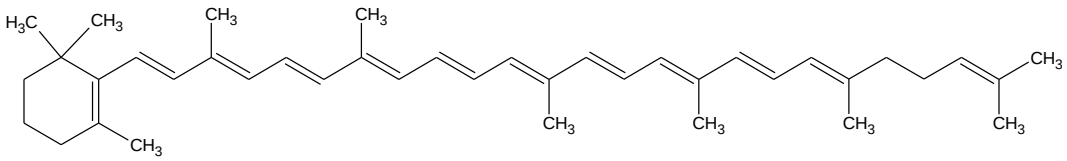

γ-Carotene

β-Carotene and Cardiovascular Disease

Cardiovascular disease (CVD) is a major cause of mortality and morbidity in the Western world. In recent years its importance has expanded internationally and it is believed that by 2020 it will be the biggest cause of mortality in the world, emphasizing the importance to prevent or minimize this increase. Studies have indicated that beta-carotene mediates pro-oxidant effects and it has been suggested that its negative effects may diminish the beneficial effects mediated by the other vitamins in the supplementation cocktail. The trials that used a combination of vitamins that include β-carotene have been disappointing (Honarbakhsh and Schachter, 2008).

In middle-aged and older women free of CVD and cancer, plasma carotenoids were associated with smoking, obesity, LDL cholesterol, HDL cholesterol, Hb A(1c), and CRP. The associations differ among individual carotenoids, possibly reflecting metabolic effects of life style and physiologic factors on plasma carotenoids, and may partially explain the inverse association of plasma carotenoids with CVD outcomes observed in epidemiologic studies.

The possible protective effect of antioxidants on coronary heart disease (CHD) has been under intensive investigation during the last two decades. The major hypothesis behind this interest is the role of oxidized low-density lipoprotein (LDL)-cholesterol in atherogenesis and the *in vitro* evidence that antioxidants inhibit oxidative modification of LDL-cholesterol (Esterbauer et al., 1989). Oxidised LDL cholesterol stimulates differentiation of monocytes into macrophages and accumulates in macrophages by a nonregulated scavenger receptor pathway. Oxidised LDL induces proliferation of smooth muscle cells, is chemotactic and cytotoxic, and impairs endothelial function (Kaplan and Aviram, 1999).

Abdominal aortic aneurysm (AAA) is a common and often a fatal condition of the human aorta with a clear male predominance. In the scope of public health, the importance of AAA has been growing, because its mortality rate has not shown a decreasing trend during the last decades (Drott et al., 1992), as has that of other cardiovascular diseases. AAA is a degenerative disorder with a complex etiology. Atherosclerosis is considered the major cause, but lately evidence of the importance of other factors has emerged. There is evidence of familial clustering and involvement of hemodynamic factors (MacSweeney et al., 1992). In patients with aneurysm, histological studies have shown atherosclerosis, inflammation, and loss of elastin and collagen content in the aortic wall (Thompson, 1996).

According to Wang et al., (2008) CVD risk factors may potentially influence plasma concentrations of carotenoids. Baseline plasma carotenoids, (α-carotene, β-carotene, β-

cryptoxanthin, lycopene, and lutein-zeaxanthin), blood lipids, Hb A(1c), and CRP were available for studies in 2895 women. The results showed that women who were current smokers or obese had lower plasma concentrations of most carotenoids expect for lycopene. An increase in LDL cholesterol was associated with a increase in α-carotene, β-carotene, and lycopene.

Tornwall et al., (2004) evaluate the 6-year post-trial effects of α-tocopherol and β-carotene supplementation on coronary heart disease (CHD). 29,133 male smokers, aged 50–69 years were randomised to receive α-tocopherol 50 mg, or β-carotene 20 mg, or both, or placebo daily for 5–8 years. At the beginning of the post-trial follow-up, 23,144 men were still at risk for a first-ever major coronary event (MCE), and 1,255 men with pre-trial history of myocardial infarction (MI) were at risk for MCE. β-Carotene seemed to increase the post-trial risk of first-ever non-fatal MI but there is no plausible mechanism to support it. So reported do not advocate the use of α-tocopherol or β-carotene supplements in prevention of CHD among male smokers.

In a nested case-control study of 513 women with cancer; 130 with cardiovascular disease and equal numbers of controls, we found no effect of randomised β-carotene on risk of cancer or cardiovascular disease within any quartile of baseline plasma β-carotene, nor was there a trend across quartiles (Lee et al., 2002).

12-year followup of cardiovascular mortality is also reported by Gey et al., (1993) and reveals a significantly increased relative risk of ischemic heart disease and stroke at initially low plasma levels of β-carotene (< 0.23 µmol/l) and/or vitamin C (< 22.7 µmol/l), independently of vitamin E and of the classical cardiovascular risk factors. Low levels of both carotene and vitamin C increase the risk further, in the case of stroke even with significance for overmultiplicative interaction. In conclusion, in cardiovascular disease independent inverse correlation may exist for every major essential antioxidant although the latter can also interact synergistically.

It is also reported the evaluate of 6-year post-trial effects of α-tocopherol and β-carotene supplementation on coronary heart disease (CHD) in the α-tocopherol, β-carotene cancer prevention (ATBC) study on 29,133 male smokers, aged 50-69 years were randomised to receive α-tocopherol 50 mg, or β-carotene 20 mg, or both, or placebo daily for 5-8 years. Results supported that α-tocopherol supplementation had no significant post-trial effect on first-ever MCEs during the 6-year followup, a result similar to that observed during the trial period. In contrast, β-carotene supplementation increased the post-trial risk of MCE (major coronary event) and non-fatal MI by 14% and 16%, respectively.

Post-trial risk for fatal CHD increased by 11%, but did not reach statistical significance. These findings of β-carotene were unexpected since no increased risk was observed during the trial period when the corresponding relative risks were 1% for MCE, 0% for non-fatal MI (myocardial infarction) and 2% for fatal CHD. The late effects of α-tocopherol and β-carotene on MCEs in men with pre-trial MI. α-Tocopherol, supplementation had no significant effect on MCEs in these men either during or after the intervention. During the intervention the risk of fatal CHD was significantly increased by 44% among those who received β-carotene compared with those who did not, whereas β-carotene had no post-trial effect on fatal CHD or non-fatal recurrent MI (Tornwall et al., 2001).

It is also reported the relation between the intakes of dietary carotene, vitamin C, and vitamin E and the subsequent coronary mortality was studied in a cohort of 5,133 Finnish men and women aged 30-69 years and initially free from heart disease. Food consumption was estimated by the dietary history method covering the total habitual diet during the previous year. Altogether, 244 new fatal coronary heart disease cases occurred during a mean follow-up of 14 years beginning in 1966-1972. An inverse association was observed between dietary vitamin E intake and coronary mortality in both men and women with relative risks between the highest and lowest tertiles of the intake (Knekt et al., 1994).

Other double-blind study were randomised to receive an antioxidant cocktail including 600 mg of α-tocopherol, 250 mg of vitamin C and 20 mg of β-carotene was supplemented for five years without any benefit on major coronary events among over 20,000 high-risk subjects (Heart Protection Study Collaborative Group, 2002). In contrast, the Cambridge Heart Antioxidant Study among 2000 patients with angiographically proven coronary atherosclerosis found a significant decrease in risk for non-fatal MI, but this surprisingly high risk-reduction was not reflected in cardiovascular mortality (Stephens et al., 1996).

Furthermore, β-carotene trials have not provided evidence of favourable effects on CHD, although the opposite was expected based on the observational studies (Pandey et al., 1995). In the Physicians' Health Study, no effect on cardiovascular mortality or risk for MI was observed among over 22,000 male physicians randomised to receive 50 mg of β-carotene or placebo every other day for 12 years (Hennekens et al., 1996). In nearly 40,000 US women randomised to receive 50 mg of β-carotene, or 600 IU of α-tocopherol, or 100 mg of aspirin, or placebo every other day no early effect of β-carotene was observed on cardiovascular endpoints (Lee et al., 1999). In these two studies, only 11% and 13% of the participants, respectively, were smokers.

In the beta-carotene and retinol efficacy trial (CARET), the effect of the combination of 30 mg of β-carotene and 25,000 IU of vitamin A supplementation on lung cancer and cardiovascular diseases was assessed among 18,000 current or former smokers or workers exposed to asbestos. A suggestion of increased risk for cardiovascular mortality was observed among those who received combination supplementation compared with those who received placebo group after an average follow-up of 4 years (Omenn et al., 1996b).

In another study, meta analysis of 6 randomised trials was observed that the risk of cardiovascular death with β-carotene treatment was slightly increased (Vivekananthan et al., 2003).

Another trial show the evaluation of the effects of α-tocopherol and β-carotene supplementation on incidence of large abdominal aortic aneurysm (AAA) in a randomised, double-blind, placebo-controlled trial. Subjects 29,133 were 50–69-years-old male smokers, participants in the Finnish α-tocopherol, β-carotene Cancer Prevention (ATBC) Study. They were randomised to receive either 50 mg/day of α-tocopherol, or 20 mg/day of β-carotene, or both, or placebo. Incidence of AAA was evaluated from mortality and hospital registers. During 5.8 years of follow-up, 181 male were diagnosed with either ruptured AAA or nonruptured large AAA treated with aneurysmectomy. A modest though nonsignificant decrease in risk for nonruptured AAA was observed among α-tocopherol and β-carotene, supplemented male compared with male not receiving these antioxidants. Neither affected

risk for ruptured AAA. In conclusion, long-term supplementation with α-tocopherol or β-carotene had no preventive effect on large AAA among male smokers (Tornwall et al., 2001).

The treatment during five year period of 20,536 UK adults (aged 40-80) with coronary disease, other occlusive arterial disease or diabetes with antoxidant vitamin supplementation (600 mg vitamin E, 250 mg vitamin C, and 20 mg of β-carotene daily). Although this regimen increased blood vitamin concentration substantially, it did not produced any significant reductions in five years mortality from, or incidence of, any type of vascular disease, cancer or other major outcome (UK Medical Research Concil, 2002).

β-Carotene and Immune Response

Carotenoids have also been shown to enhance immune responses. Both T and B lymphocytes in the spleens of rats fed nutritionally complete diets supplemented with either β-carotene or canthaxanthin show enhanced proliferative responses (Bendich, 1989a). Hamsters bearing chemically induced tumors that were treated with β-carotene, canthaxanthin, or other carotenoid preparations showed increased numbers of cytotoxic T cells and macrophages as well as higher titers of tumor necrosis factor than did untreated animals. Mice that were fed β-carotene, canthaxanthin, or astaxanthin and then injected with tumor cells developed fewer and more slowly growing tumors than those that were not given carotenoids (Bendich and Shapiro, 1986). The mechanism by which carotenoids are producing these effects on the imumune system, however, is not yet clear.

β-Carotene as Anti-Inflammatory

β-Carotene has shown antioxidant and anti-inflammatory activities; however, its molecular mechanism has not been clearly defined. Bai et al., (2005) examined *in vitro* and *in vivo* regulatory function of β-carotene on the production of nitric oxide (NO) and PGE2 as well as expression of inducible NO synthase (iNOS), cyclooxygenase-2, TNF-α, and IL-1β. β-Carotene inhibited the expression and production of these inflammatory mediators in both LPS stimulated RAW264.7 cells and primary macrophages in a dose-dependent fashion as well as in LPS-administrated mice.

Furthermore, this compound suppressed NF-κB activation and iNOS promoter activity in RAW264.7 cells stimulated with LPS. β-Carotene blocked nuclear translocation of NF-κB p65 subunit, which correlated with its inhibitory effect on IκBα phosphorylation and degradation. This compound directly blocked the intracellular accumulation of reactive oxygen species in RAW264.7 cells stimulated with LPS as both the NADPH oxidase inhibitor diphenylene iodonium and antioxidant pyrrolidine dithiocarbamate did. The inhibition of NADPH oxidase also inhibited NO production, iNOS expression, and iNOS promoter activity. These results suggest that β-carotene possesses anti-inflammatory activity by functioning as a potential inhibitor for redox-based NF-κB activation, probably due to its antioxidant activity.

Some investigators found the redox effect of β-carotene previously observed *in vitro* was also observed *in vivo* (Imamura et al., 2006). Ingested β-carotene elicited an increase in GSH in murine splenocytes, accompanied by an increase in mRNA for γ-GCS. The amount of intracellular glutathionedisulfide (GSSG), an alternative form of glutathione, is far less than that of intracellular GSH in mammalian cells (Droge et al., 1994). Enhanced transcription of γ-GCS by β-carotene in a cultured cell line has been reported by some investigators (Ben-Dor et al., 2005). The increases in intracellular GSH found by Takeda et al., (2008) might be attributable to the reinforced production of GSH induced by β-carotene. The health benefits of β-carotene, other than as a provitamin A, are controversial, but the results suggest that ingested β-carotene has a positive effect on the redox status of immune cells.

β-Carotene as Antioxidant

About 20 years ago the hypothesis that diet might have a substantial influence on the development of some pathologies, such as cancer, has been raised by many scientists. In this light, during the last decade, efforts have been made to analyze the effects of plant food and synthetic antioxidants on the development and prevention of chronic diseases. Nowadays, antioxidants are used on a large scale to try to obtain and preserve optimal health.

While there is no doubt that the correct balance between endogenous and exogenous antioxidant capacity is essential to life, the curative power of antioxidants has often been overestimated. In fact, according to the popular idea "if one is good two is better", antioxidants are taken in excess too often and the risk to originate diseases instead of preventing them is quite high. It is noteworthy to underlie that as for all drugs, antioxidants may give important side effects if not correctly used or in combination with other drugs. Vitamin A, E and β-carotene for instance, have been shown to have pro-oxidant effects at higher doses or under certain conditions (Lopez-Hellin et al., 1998).

Oxidative stress is implicated in most human diseases. Antioxidants may decrease the oxidative damage and its alleged harmful effects. Carotenoids may protect cells from oxidative stress by quenching free radicals capable of causing cellular damage. Unsaturated lipids in cell membranes are prime targets for free radical reactions. A free radical-mediated attack on lipid membranes can initiate a chain reaction that results first in lipid peroxidation and ultimately in functionally significant damage to membranes, enzymes, and nucleic acids. Both *in vivo* and *in vitro*, β-carotene has been shown to protect isolated lipid membranes from peroxidation, LDL-containing lipids from oxidation, and liver lipids from oxidation induced by carbon tetrachloride-induced free radicals.

In chemical studies, the possible basis for the protective actions of carotenoids has been examined. Although β-carotene primarily has been studied, theoretically all carotenoids with a similar conjugated double bond system should act similarly (Krinsky and Deneke, 1982). In purely chemical studies, β-carotene interacts with peroxyl radicals irreversibly to form a carbon-centered carotenoid radical. This carbon-centered radical is resonance stabilized to such a degree that its subsequent reaction with molecular oxygen to form a peroxy-β-carotene radical is reversible (Burton,1989).

When the oxygen tension is low, the concentration of the highly reactive peroxy-β-carotene radical is reduced. The less reactive β-carotene radical can also undergo termination by reaction with another peroxy radical (Burton, and Ingold,1984). It is difficult to extrapolate directly from chemical to biological systems. Cells contain a, complex set of molecules and enzyme systems for protection from oxidative stress (Halliwel1,1988). Because free radical reactions are essential for maintaining life, the key issue is the maintenance of an appropriate balance between peroxidative events that are necessary and those that are excessive (Bendich and Olson, 1989).

Many people are taking antioxidant supplements, believing to improve their health and prevent diseases (Balluz et al., 2000). Whether antioxidant supplements are beneficial or harmful is uncertain (Herbert,1997). Many primary or secondary prevention trials of antioxidant supplements have been conducted to prevent several diseases.

In addition, conclusions on beneficial effect of antioxidant are often drawn from studies conducted with synthetic antioxidant supplement, whereas fruits and vegetable are a complex mixture of antioxidant, as well as other potentially beneficial micronutrients and macronutrients, which may, thus, work with different kinetics and dynamics (Bardia et al., 2008).

In conclusion, the correct use of antioxidants may be useful to prevent free radical-related disorders. However, the repair of existing critical structural damage may be beyond the possibilities of antioxidants and therefore they may not be considered to be useful in therapeutic clinical applications, where their limits and eventual side effects must be better understood (Brambilla et al., 2008).

Some investigators analyze the effects of antioxidant supplements as selenium, beta carotene, vitamins A, E, and C. With exception of selenium, the others compounds showed no significant effects on gastrointestinal cancers (Bjelakovic et al., 2004). It is also reported that no beneficial or harmful effect or significantly increased mortality of these supplements ((Bjelakovic et al., 2006; Caraballoso et al., 2003; Davies et al., 2006; Huang et al., 2006). In addition, an study included 68 randomized trials with 232,606 participants involving adults comparing beta carotene, vitamin A, vitamin C, vitamin E, and selenium either singly or combined vs placebo or vs no intervention were included in the analysis. Results supported an increased mortality of about 5% is likely to be conservative. Treatment with beta carotene, vitamin A, and vitamin E may increase the risk of death (Bjelakovic et al., 2007).

β-Carotene and Skin Photodamage

Ultraviolet (UV) radiation is one of the most abundant carcinogens in our environment, and the development of non-melanoma skin cancers, the most common type of human malignancy worldwide, represents one of the major consequences of excessive exposure. Because of growing concerns that the level of UV radiation is increasing as a result of depletion of the stratospheric ozone and climate change, the development of strategies for protection of the skin is an urgent need. Many phytochemicals that belong to various families of secondary metabolites, such as alkaloids (caffeine, sanguinarine), flavonoids [(-)-epigallocatechin 3-gallate, genistein, silibinin], carotenoids (beta-carotene, lycopene), and

isothiocyanates (sulforaphane), offer exciting platforms for the development of such protective strategies. These phytochemicals have been consumed by humans for many centuries as part of plant-rich diets and are presumed to be of low toxicity, an essential requirement for a chemoprotective agent. Mechanistically, they affect multiple signalling pathways and protect against UV radiation-inflicted damage by their ability to act as direct and indirect antioxidants, as well as anti-inflammatory and immunomodulatory agents (Dinkova-Kostova, 2008).

Skin cancer is a major public health issue in white-skinned populations in the United States, Europe, and Australia, and the incidence continues to rise (Staples et al., 1998). Solar keratoses (SKs) are among the strongest determinants of skin cancer risk. The risks of the main types of skin cancer-basal cell carcinoma and squamous cell carcinoma (BCC and SCC) are increased 3- to 12-fold in the presence of SKs (Marks et al., 1988). Indeed, a high proportion of SCCs are believed to arise in SKs, although the actual rate of transformation is small (Frost et al., 2000). Despite the possibility that controlling SK development may effectively reduce skin cancer.

The effect of sunscreen (application of a high-protection sunscreen to their head, neck, arms, and hands every morning) or application of sunscreen at their usual discretionary rate was determinate in an randomized controlled trial conducted between 1621 adults aged 25 to 74 years. They were also randomly assigned to take either one 30-mg tablet of beta carotene or one placebo tablet each day.

The results showed a reduction in the rate of change of SK prevalence was also seen in the sunscreen intervention group relative to the discretionary sunscreen group between 1994 and 1996, but it was not significant. No effect on the rate of change of prevalent SK counts was seen among those taking beta carotene supplements relative to those taking placebo tablets. Daily application of sunscreen retarded the rate of SK acquisition among adults in a subtropical environment, while a beta carotene supplementation of 30 mg/day had no influence on the occurrence of SKs (Darlington et al., 2003).

β -Carotene has been extensively investigated as a chemopreventive agent that may protect against skin photodamage (Mathews-Roth, 1990). Recently, β-carotene has been used as a component of some cosmetics (Harang, 2000.) However, inconsistent findings exist in various studies (Garmyn et al.,1995). For example, Black (Black, 1998.) found that β-carotene-supplemented semidefined diets, in contrast to commercial closed formulas, not only fail to protect against UV-induced carcinogenesis but also lead to significant exacerbation in mice. Black et al. (2000) pointed out that the inconsistency in the photoprotective effect of β-carotene in animal studies may have been due to the interaction between β-carotene and other dietary antioxidants including phytochemicals. Similarly, an *in vitro* study showed that preincubation of skin fibroblasts with either β-carotene or lycopene (0.1–1.0 μM) increases ultraviolet A (UVA)-induced expression of metalloproteinase 1 (MMP-1) (Offord et al., 2002), a collagenase associated with skin aging, while concurrent addition of vitamin E or vitamin C during preincubation suppresses the increase in MMP-1 mRNA. However, limited data exist regarding the interaction of β-carotene with other phytochemicals on UVA-induced oxidative damage.

Flavonoids are a major type of phytochemicals that are ubiquitously present in fruits and vegetables. Growing evidence demonstrate an inverse relation between the dietary intake of

flavonoids and the incidence of several chronic diseases and cancers (Knekt et al., 2002). In addition, flavonoids may protect against photodamage (Sies and Stahl, 2004). These beneficial effects of flavonoids have been attributed to their actions as antioxidants, enzyme inhibitors and cell cycle regulators under various conditions (Depeint et al., 2002). An *in vitro* study showed that flavonoids, including naringenin, rutin and flavonoid extracts from apple skin, prevent UVB-induced DNA damage.

Yeh et al., (2005) demonstrates an interaction between flavonoids and β-carotene in UVA-induced DNA damage. Researchers suggest that a combination of β-carotene with naringin, rutin or quercetin may increase the safety of β-carotene.

Photooxidative stress may play a role in the etiology of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), collectively termed nonmelanoma skin cancer (NMSC). Antioxidant vitamins (e.g., β-carotene) might therefore offer some protection (Steenvoorden and van Henegouwen,1997). β-carotene might also prevent UV-induced immunosuppression (Herraiz et al., 1998), enhance cell-to-cell communication (Zhang et al., 1991), and affect cell proliferation and differentiation (Khuri et al., 1997). Animal studies of UV induced skin cancer have provided consistent evidence of an anticancer effect of carotenoids (IARC, 1998a). In contrast, randomized trial data show no effect of β-carotene supplementation on NMSC (Greenberg et al., 1990; Green et al., 1999).

A nested case-control study was conducted within the Physicians' Health Study, a randomized, double-blind, placebo-controlled trial of 50 mg β-carotene supplementation on alternate days with 12 years of follow-up. Study subjects were 1,338 men ages 40 to 84 years at baseline who, during follow-up, developed a NMSC (including 1,156 with BCC and 166 with SCC) and an age and smoking-matched control group of 1,338 men who remained free of NMSC at the time of diagnosis of the case.

This study provides substantial evidence that there is no beneficial effect of 12 years of β-carotene supplementation on the risk of NMSC, including BCC and SCC, among subjects with the lowest baseline plasma levels of β-carotene, α-tocopherol, or vitamin A. Furthermore, there is no association between plasma levels of β-carotene, α-tocopherol, or vitamin A and risk of NMSC. Until further evidence emerges, risk reduction through limiting exposure to UV light remains the best available strategy for prevention of NMSC. (Schaumberg et al., 2004).

Mathews-Roth (1982) so reported that the administration of β-carotene caused a delay in the appearance of and decreased in the number of skin tumors in hairless mice induced by UV radiation.

β-Carotene and Diabetes

Carotenoids demonstrate a vast array of biological activities, including vital roles in the eye, both functionally as precursors to retinol in the visual pathway (pro-vitamin A (PVA) carotenoids) and structurally as macular pigments. The major PVA carotenoids in plasma are α-carotene, β-carotene and β-cryptoxanthin. of these, only β-carotene is found in ocular tissues (Krinsky and Johnson, 2005).

In contrast, lutein/zeaxanthin and lycopene are the major non-PVA carotenoids, i.e. are not retinol precursors, and both are present in ocular tissues at high concentrations. Lutein and zeaxanthin comprise the macular pigments, essential for normal vision and for the protection of photoreceptors from phototoxic blue light, while lycopene is present in high concentrations in the human ciliary body and retinal pigment epithelium/choroids (Khachik et al., 2002). Plasma carotenoid concentrations have been linked to numerous conditions (Voutilainen et al., 2006.) including the major blinding conditions–age-related macular degeneration (Cardinault et al., 2005) and cataracts (Gupta et al., 2003).

Consequently, Laima et al., (2008) undertook to investigate the association between plasma carotenoids and diabetic retinopathy. 64 diabetic retinopathy participants (range 44–77years) was significantly associated with established risk factors, i.e. duration of diabetes, HbA1c, use of hypoglycaemic medication, and the albumin excretion rate. A longer duration of diabetes was, however, the only independent predictor of diabetic retinopathy, as demonstrated by multivariate modelling of these factors. The observed α-carotene concentrations were at the higher end of values reported in other diabetic populations and were associated with diabetic retinopathy. Conversely, lycopene, a non-PVA carotenoid, demonstrated a trend to lower levels in the retinopathy group. In addition, a higher plasma non-PVA:PVA carotenoid ratio was inversely associated with diabetic retinopathy.

In conclusion, synergies between plasma carotenoids seem to be implicated in diabetic retinopathy, independent of established risk factors. In general, the research provides additional data concerning the importance of carotenoid-rich foods for health maintenance and gives strength to the recommendation of increasing consumption of lutein- and lycopene-rich foods.

Glucose-intolerant states are now thought to be characterized by increased oxidative stress, as demonstrated by increased reactive oxygen species and lipid peroxidation, and increased free radical activity (Gupta et al., 2003a). Several mechanisms including autoxidation, glycation, the polyol pathway, and activation of monocytes have been proposed to account for the increase in reactive oxygen species. Oxidative stress can result in the lowering of antioxidant concentrations in people with glucose intolerance. Thus, it is conceivable that both endogenous and exogenous antioxidants could play a role in the pathogenesis of glucose intolerance. Carotenoids are one possible source of exogenous antioxidants. However, little is known about the association of individual serum carotenoid concentrations and glucose intolerance.

Ford et al., (1999) evaluate the associations between glucose intolerance and serum α-carotene, β-carotene, cryptoxanthin, lutein/zeaxanthin, and lycopene in 4,423 participants between 40 and 74 years old, 1,958 participants attended the morning clinic session. These population-based data from NHANES III suggest that carotenoid concentrations are associated with insulin resistance and glucose tolerance status.

However, the cross-sectional nature of the data limits inferences on temporality and causation. The evidence was strongest for β-carotene and lycopene, which showed linear relations with the degree of glucose tolerance abnormality. Cryptoxanthin also was lower in persons with newly diagnosed diabetes compared with persons with a normal glucose tolerance, α-carotene and lutein/zeaxanthin were not significantly different among participants with abnormal glucose tolerance compared with those with a normal one.

Previously, diabetes was shown to be inversely related to β-carotene but not α-carotene, β-cryptoxanthin, or lutein in a study of 109 dialysis patients (Rock et al., 1997). All the carotenoids were inversely related to fasting insulin concentration, supporting an association between serum carotenoid concentrations and insulin resistance and thus raising the possibility that carotenoids may favorably affect glucose tolerance by influencing insulin resistance.

Oxidative stress increases during pregnancy and birth. The high metabolic rate of the placenta leads to increased generation of free radicals, biomarkers of which were observed in maternal circulation (Chen and Scholl, 2005). The concentration of lipoperoxides in cord blood is only30%of that in maternal blood, suggesting that the placenta suppresses lipoperoxide formation or transplacental passage, protecting the fetus from the action of these free radicals (Takehara et al., 1990). At birth, the neonate faces an increase in oxidative aggression and presents high concentrations of hydroperoxides in erythrocyte membranes, indicating oxidative stress (Robles et al., 2001).

Since the fetal origins hypothesis was widely introduced in the 1980s and 1990s (Godfrey and Barker, 2001), increased attention has been paid to the fetal period in search for the causes of chronic illnesses, including type 1 diabetes (Lindberg et al., 1999). Maternal dietary nitrite intake was associated with increased risk of type 1 diabetes in the offspring (Virtanen et al., 1994), and a strong inverse association was reported between maternal use of cod liver oil during pregnancy and the risk of type 1 diabetes in the child. Perinatal events were related to an increased risk of childhood type 1 diabetes (Dahlquist et al., 1999).

Several studies were carried out as part of the population-based birth cohort of the Type 1 Diabetes Prediction and Prevention Project. The data comprised 4297 children with increased genetic susceptibility to type 1 diabetes, born at the University Hospital of Oulu or Tampere, Finland between October 1997 and December 2002. The children were monitored for diabetes-associated autoantibodies from samples obtained at 3-12-mo intervals. Maternal antioxidant intake during pregnancy was assessed postnatally with a self-administered food-frequency questionnaire, which contained a question about consumption of dietary supplements. Maternal intake of none of the studied antioxidant nutrients showed association with the risk of advanced β cell autoimmunity in the child. The hazard ratios, indicating the change in risk per a 2-fold increase in the intake of each antioxidant, were nonsignificant. Results supported the hhigh maternal intake of retinol, β-carotene, vitamin C, vitamin E, selenium, zinc, or manganese does not protect the child from development of advanced β cell autoimmunity in early childhood (Uusitalo et al., 2008).

It was also reported in another study the ability of the micronutrients as β-carotene to reduce the risk of development of age-related macular degeneration in the Age-Related Eye Disease Study (AREDS) can have the same effect on the development of diabetic retinopathy in rats (Adelman, 2001).

In addition, the treatment of the streptozotocin-induced diabetic rats with a powdered diet with or without supplemental micronutrients (ascorbic acid, vitamin E, beta-carotene, zinc, and copper) was studied. The retina was used after the rats had diabetes for 12 months to detect vascular histopathology and to measure the biochemical parameters and messenger RNA levels of the genes involved in oxidative and nitrative stress. The AREDS-based micronutrients prevented a diabetes-induced increase in the number of retinal acellular

capillaries. In the same rats, micronutrients inhibited increases in retinal oxidatively modified DNA and nitrotyrosine and decreases in manganese superoxide dismutase. Diabetes-induced alterations in the messenger RNA expression of mitochondrial electron transport complex III (coenzyme Q cytochrome-c reductase) and inducible nitric oxide synthase were also prevented. Age-Related Eye Disease Study-based micronutrients inhibit the development of diabetic retinopathy in rodents by inhibiting oxidative and nitrative stress (Kowluru et al., 2008).

Beta-carotene supplements do not appear to lower the risk of developing type 2, or adult-onset, diabetes. Researchers analyzed the development of type 2 diabetes in 22,071 healthy US male physicians aged 40 to 84 years in a randomized, double- blind, placebo-controlled trial, from 1982 to 1995 participating in the Physicians' Health Study, about half of whom were taking beta-carotene supplements (50 mg on alternate days) or placebo. Over a 12-year period, the rate of type 2 diabetes was similar in men who took beta-carotene and those who did not (Liu et al., 1999).

β-Carotene and Maculopathy

In the USA, an estimated 30% of persons 65 years and older show signs of early age related maculopathy (ARM) or its late-stage manifestations, which are also known as age-related macular degeneration (AMD) (National Advisory Eye Council, 2004).

In a randomized trial conducted for a 12 years' period of duration from a large population 22, 071 apparently healthy US male physicians aged 40 to 84 years. Participants were randomly assigned to receive beta carotene (50 mg every other day) or placebo. However, the main trial results indicated that 12 years of randomized β-carotene treatment had no beneficial or harmful effect on any cancer or cardiovascular disease end point in the overall population or in the 11.0% of physicians who were current smokers at baseline (Hennekens et al., 1996). Two other randomized trials also reported no effect of beta carotene supplementation on cancer, cardiovascular, or mortality end points (Greenberg et al., 1996; Lee et al., 1999).

In summary, the results of this trial indicate that beta carotene supplementation for 12 years has little effect on the development of visually significant ARM in apparently healthy men (Christen et al., 2007).

β-Carotene and Pregnancy

Reducing infant mortality remains a major public health challenge in developing countries. In recent years, attention has turned to reaching newborns with safe, efficacious interventions to improve survival (Martines et al., 2005). Long known to reduce child mortality over 6 months of age (Sommer and West, 1996), new observations have emerged in recent years that vitamin A, if given as an oral supplement shortly after birth, can reduce infant mortality. Two randomized, placebo-controlled trials in South Asia have, to date, reported significant reductions in infant mortality after receipt of a large, oral dose of vitamin

A (~50 000 IU) within several hours to several days after birth. In Indonesia, Humphrey et al (1996) reported a 64% reduction in mortality in a trial among 2067 hospital-born infants, and in south India, Rahmathullah et al (2003) observed a 22% reduction in mortality through 6 months of age in a community-based trial among 11,619 infants.

Vitamin A deficiency is common among women in developing countries. Mean serum retinol concentrations of about 1.05 µmol/l (300 µg/l) have been reported during pregnancy among diverse groups of south Asian women (Sivakumar et al., 1997) in comparison with values of 1.57-1.75 µmol/l (450-500 µg/l) in better nourished populations (Morse et al., 1975).

Concern about maternal vitamin A deficiency has focused on its effects on fetal and infant vitamin A status, health, and survival, with little attention being paid to its effects on the health consequences for the woman (Dimenstein et al., 1996). An early trial in England reported that maternal vitamin A supplementation in late pregnancy through the first week post partum could reduce the incidence of puerperal sepsis (Green et al., 1931), but this lead was ignored.

In Nepal maternal night blindness, an indicator of vitamin A deficiency, has been associated with increased risks of urinary or reproductive tract infections and diarrhoea or dysentery and raised acute phase protein concentrations during infection (Christian et al., 1998) That vitamin A deficiency could predispose women to increased infectious morbidity and mortality is supported by evidence in children and animals. Mechanisms underlying such an effect could include impaired barrier defences of epithelial tissues and compromised innate and acquired immunity (Semba, 1994). Conducted a study in rural southeast central plains of Nepal to assess the impact on mortality related to pregnancy of supplementing women of reproductive age each week with a recommended dietary allowance of vitamin A, either preformed or as β-carotene. The treatment of the 44,646 married women, of whom 20,119 became pregnant 22,189 times to receive weekly a single oral supplement of placebo, vitamin A (7000 µg retinol equivalents) or β-carotene (42 mg, or 7000 µg retinol equivalents) for over 3½ years. Supplementation of women with either vitamin A or β-carotene at recommended dietary amounts during childbearing years can lower mortality related to pregnancy in rural, undernourished populations of south Asia (West et al., 1999).

In other trail conducted in 19 unions in rural northwest Bangladesh was evenly randomized for newborns of participating mothers to receive a single, oral supplement of vitamin A (50 000 IU) or placebo as droplets of oil squeezed from a gelatinous capsule. Mothers provided informed consent for newborn participation at ~28 weeks gestation. After birth, typically at home, 17,116 infants were supplemented and their vital status was followed through 24 weeks of age. The main outcome measure was mortality through 24 weeks of age. Newborn vitamin A dosing improved infant survival through the first 6 months of life in Bangladesh. These results corroborate previous findings from studies in Indonesia and India and provide additional evidence that vitamin A supplementation shortly after birth can reduce infant mortality in South Asia (Klemm et al., 2008).

β-Carotene and Cancer

There is a strong evidence from observational epidemiology that fruits and vegetables in the diet are associated with a lower incidence of various cancers. From this has developed the idea that it is the antioxidants in these foods that are the effective preventive agents. This is an attractive hypothesis; it is known that free radicals released during respiration can damage DNA, that oxidation damage to DNA can result in mutation, and that fruits and vegetables contain substantial amounts of various natural compounds with antioxidant properties. However, confirmation of this hypothesis remains an elusive goal of experimental scientists as well as conventional epidemiologists.

Low intake of vegetables and fruits and carotenoids is consistently associated with an increased risk of lung cancer in both prospective and retrospective studies. In addition, low levels of serum or plasma β-carotene are consistently associated with the subsequent development of lung cancer. Retinol is not related in a similar manner to lung cancer risk, β-carotene seems to play a role that does not require its conversion into vitamin A. In addition, smoking, a powerful risk factor for lung cancer, is associated with reduced intake of carotenoids and lowered blood levels of β-carotene and has not always been adequately controlled in these analyses (Ziegler, 1989).

Is not know how the fruits and vegetables protect against cancer, but it seems increasingly unlikely that it is simply because they contain high concentrations of antioxidants. Also to consider the effects of phytochemicals (which mayor may not be antioxidants) on many other cellular functions, including cell-signalling, apoptosis, antioxidant enzymes, the phase I and II xenobiotic-metabolising enzymes, DNA repair, plus of course, the enormous potential for effects on gene expression that might have an impact on the carcinogenic process.

It was probably Ames (1983) who first drew general attention to the importance of oxidative damage in human cancer aetiology and the likely importance of antioxidant defences, both intrinsic (glutathione, uric acid, superoxide dismutase, etc.) and of dietary origin. He surveyed the large number and variety of natural chemicals in plants used as human food that have been shown to be mutagenic (i.e., capable of causing mutations *in vitro* tests such as the Ames test) or carcinogenic (inducing cancer in animals), or both. Many of these may act through the generation of oxygen free radicals. Perhaps, as Ames implies, we are protected against deleterious effects of oxygen free radicals by the antioxidants and other anticarcinogens that are also present in plant-derived foods. These phytochemicals would also protect us against the effects of endogenous production of reactive oxygen, as a by-product of normal respiration, as part of the inflammatory response, or during xenobiotic metabolism.

In the case of DNA oxidation, it is possible to demonstrate a decrease in oxidative damage after supplementation with isolated antioxidants or whole plant foods in humans. In contrast, in several large-scale interventions with disease or death as the endpoint, supplementation with β-carotene resulted in no effect or an increase in cancer incidence. It is certainly true that we do not yet fully understand the role of phytochemicals as antioxidants, or as modulators of other processes related to carcinogenesis and its prevention (Collins, 2005).

As a consequence of these contradictory findings, there has been considerable interest in elucidating the mechanism(s) by which β-carotene may act as a pro- and/or anticancerogenic agent in humans. It has been suggested that β-carotene may behave as an intracellular redox agent, acting as an antioxidant (Palozza and Krinsky, 1992) in some circumstances and as a pro-oxidant in others (Palozza P. 1998).

In particular, at doses of β-carotene that exceed the normal dietary intake and in conditions of enhanced oxidative stress, such as those found in tumor cells and in normal cells exposed to tobacco smoke, the carotenoid may act as a propagator of free-radical formation. It has been suggested that several pro- and anti-tumor agents affect cell growth by modulating apoptosis (Raff, 1992), oxidative stress has been suggested to play a key role as a mediator of apoptosis (Jacobson, 1996.). Moreover, it has been suggested that Bcl-2, a protein blocking apoptosis, inhibited cell death by reducing the generation of reactive oxidants, thereby preventing a critical intracellular oxidation (Kane et al., 1993).

Furthermore, provides evidence for a possible mechanism by which β-carotene regulates cell growth. In particular, in a malignant human colon cell line, was demonstrated that β-carotene at high concentrations can act as a modulator of intracellular ROS production and that such a modulation can modify cell growth by affecting molecular pathways involved in apoptosis (Palozza et al., 2001). The ability of β -carotene and other carotenoids to inhibit tumor cell growth has been previously reported *in vivo* and *in vitro*. β-carotene was able to block tumor incidence and progression in animal models (Gerster, 1995) and to reduce the growth of tumor cell lines, including colon (Iftikhar et al.,1996), melanoma (Hazuka et al., 1990), prostate (Williams et al., 2000), oral, lung, and breast (Schwartz and Shklar, 1992) cancer cells. A wide range of β-carotene concentrations (1-300 mM) was used in culture studies to demonstrate growth-inhibitory effects of β-carotene (Touvier et al., 2005).

Cancer Prostate

There is increasing evidence that systemic oxidative stress plays an important role in the development and progression of cardiovascular disease and cancer (Wartenberg, 2005). Oxidative stress is defined as a state in which the level of toxic reactive oxygen intermediates (free radicals) overcomes the endogenous antioxidant defenses of the host such as the lipid-soluble antioxidants including vitamins A, E and the carotenoids. Oxidative stress can result, therefore, from either an excess in oxidant production or depletion of antioxidant defenses (Ames, 1988; Arnes, 1989). In the absence of adequate levels of lipid-soluble antioxidants, increased free radical production may cause functional and structural damage by reacting with lipoproteins, resulting in lipid peroxidation with the formation of degradation products, such as malondialdehyde, which are themselves carcinogenic (Tribble et al., 1976; Tribble et al., 1987; Gutteridge, 1995).

In particular, the carotenoid lycopene is one of the most potent antioxidants found in human plasma. However, plasma concentrations appear to better reflect prostatic exposure than self-reported usual dietary intake (Freeman et al., 2000). Both the tumor growth and the systemic inflammatory response have the potential to produce reactive oxygen intermediates or oxygen free radicals and thus increase oxidative stress. Indeed, both the presence of cancer

and the systemic inflammatory response are associated with lower carotenoids concentrations *(Talwar et al., 1997).*

It is therefore of interest that patients with prostate cancer have been reported to have low lycopene and β-carotene and increased oxidation of serum lipids and proteins. Indeed, patients with prostate cancer fed lycopene enriched supplement prior to prostatectomy appear to show reduced oxidative stress and tumor growth. However, a proportion of patients with prostate cancer will have evidence of a systemic inflammatory response, and its effect on carotenoids concentrations is not clear (*Rao et al., 1999*).

Almushataf et al., (2006) observed that in patients with benign prostate hyperplasia (BPH) were older had higher malondialdehyde concentrations and lower circulating concentrations of lutein, lycopene and β-carotene. Patients with metastatic prostate cancer had a higher Gleason score, but lower concentrations of α-tocopherol, retinol, lutein, β-carotene and lycopene.

Colon Cancer

Colorectal cancer remains the second commonest cause of cancer deaths in Western Europe and North America. Each year in the United Kingdom, there are ~ 35,000 new cases and 16,000 deaths attributable to the disease (Cancer Research, 2006). Overall survival is poor; even in those patients who undergo potentially curative resection, more than one-third die within 5 years (McArdle and Hole, 2002). It is increasingly recognized that variations in outcome in cancer patients are not solely determined by the characteristics of the tumor but also by host-immune response factors (MacDonald, 2007). It is now accepted that the host systemic inflammatory response can be assessed by examining the changes in the circulating concentrations of acute-phase proteins, such as an elevated concentration of C-reactive protein and a low concentrations of albumin and that these have prognostic values in patients with cancer (McMillan et al., 2000). Recently, the combination of C-reactive protein and albumin, known as the Glasgow prognostic score (GPS), has been validated as a prognostic factor in patients with colorectal cancer (McMillan et al., 2007).

The tumor growth and progression and the systemic inflammatory response have the potential to produce free radicals and thus increase oxidative stress. Indeed, both the presence and progression of cancer (Rasheed et al., 2007) and the systemic inflammatory response (Talwar et al., 1997) are associated with lower carotenoid concentrations. However, to date the majority of studies have only included relatively small number of cancer patients (Thurnham et al., 1986).

Colon cancer and its occurrence is commonly ascribed to the transformation of normal colon epithelium to adenomatous polyps and ultimately invasive cancer (Parker, 1996). According to the model proposed by Fearon and Vogelstein, cancer develops as a consequence of genetic alterations which accumulate over one or two decades (Fearon and Vogelstein, 1990). Experimental and epidemiological data have linked dietary composition with colorectal carcinogenesis. In particular, evidence from recent epidemiological studies has shown that a high dietary intake of fruit and vegetables, rich in β-carotene and other

carotenoids, is associated with a low risk for colon neoplasia (Giovannucci et al., 1992, Slattery et al., 2000).

Although some human intervention trials failed to demonstrate prevention of colon cancer by β-carotene supplements (Greenberg et al., 1994), an extensive intervention study in China showed a significant protective effect of β-carotene in combination with vitamin E and selenium on gastrointestinal cancer in a population at high risk (Taylor et al., 1994). Moreover, β-carotene supplementation reduced the rate of colon cell proliferation in patients with adenomatous polyps. Interestingly, the carotenoid has been reported to accumulate in colonic neoplastic tissues in humans and several experimental studies suggest that it can be used to enhance cytotoxicity of chemotherapeutics (Phillips et al., 1993).

Concomitantly, several reports have shown protective effects of β-carotene against colon carcinogenesis in animal models and growth inhibitory effects by carotenoids were also observed in human colon cancer cell lines (Temple and Basut, 1989). The mechanism by which β-carotene may protect from colon cancer is poorly understood. Palozza et al., (2001) recently reported that β-carotene was able to inhibit the growth of a human colon adenocarcinoma cell line through a mechanism involving apoptosis induction. In the same cell line we also demonstrated that this effect was independent of pro-vitamin.

Also Palozza et al., (2002) demonstrates that β-carotene, inhibits the growth of several human colon adenocarcinoma cell lines (COLO 320 HSR, LS-174, HT-29 and WiDr) by inducing cell cycle arrest in G_2/ phase and apoptosis. These effects were dose and time dependent and strictly related to cell ability to accumulate the carotenoid. COLO 320 HSR cells incorporated β-carotene to a greater extent than LS-174, HT-29 and WiDr cells and, concomitantly, they exhibited a higher sensitivity to the growth inhibitory effects of the carotenoid. At inhibitory concentrations β-carotene reduced the expression of cyclin A, a key regulator of G2/M progression. Neither p21 nor p27, two cyclin kinase inhibitors, were significantly modified by carotenoid treatment. With respect to apoptosis induction, decreased levels of the apoptosis blocking proteins Bcl-2 and Bcl-xL were also observed.

Accumulating evidence suggests that colorectal tumorigenesis may be regulated by cyclooxygenase (COXY-2), an inducible enzyme responsible for the conversion of arachidonic acid to prostaglandins (Prescott and White, 1996). Tsujii and DuBois (1995) first reported that cells expressing high levels of COX-2 had increased tumorigenic potential that could be reversed by COX-2 inhibitors. Using an APC knockout mouse model, Oshima et al. (1996) demonstrated that COX-2 expression was induced very early in neoplastic progression. Interestingly, the number and size of intestinal polyps were dramatically reduced by specific COX-2 inhibitors.

Moreover, recent studies showed increased levels of COX-2 in colorectal carcinomas compared with adjacent normal appearing mucosa (Sano et al., 1995). COX-2 expression is induced by growth factors such as epidermal growth factor (EGF) or tumor growth factor-α in a number of cell systems, including rat intestinal epithelial cells (DuBois et al., 1994) and HCA- 7 colon cancer cells (Coffey et al., 1997). High levels of this, protein have been associated with a decreased ability of cells to undergo apoptosis, suggesting that COX-2 expression may protect cancer cells from apoptosis induced by a variety of stimuli and could enhance cell tumorigenic potential (Richter et al., 2001). Thus, it has been suggested that COX-2 inhibitors are also able to act as apoptotic inducers (Elder et al., 1999).

Recently, much attention has been devoted to identifying colon cancer chemopreventive agents of dietary origin (Surth, 1999). Moreover, β-carotene supplementation reduced the rate of colon cell proliferation in patients with adenomatous polyps (Cahill et al., 1993). Concomitantly, protection by β -carotene against colon cancer was shown in animal models (Alabaster et al., 1995) as well as in cultured cells. In particular, was observed recently that β-carotene arrested the growth of different human colon adenocarcinoma cells in a manner strictly related to the cell's ability to accumulate the carotenoid and by a mechanism involving both cell cycle arrest and induction of apoptosis (Iftikhar et al., 1996).

Taken together, these data raise questions about the possibility that the growth-inhibitory and proapoptotic effects of β-carotene observed in experimental and clinical studies may involve a reduction in the expression of COX-2. Therefore, Palozza et al., (2005) verifying the effect of β-carotene on the growth of human colon adenocarcinoma cells overexpressing (LS-174, HT-29, WiDr) or not expressing (HCT116) COX-2, COX-2 expression induced by the growth factor heregulin-α, which promotes COX-2 expression through the stimulation of HER2/HER3 receptors, and induction of apoptosis. The downregulation of COX-2 by β-carotene occurred in both untreated and heregulin-treated cells. It was accompanied by an increased ability of cells to undergo apoptosis and by a decrease in intracellular ROS production and in the activation of ERK1/2. Moreover, cells not expressing COX-2 were insensitive to the growth-inhibitory and proapoptotic effects of the carotenoid. The suppression of COX-2 by β-carotene may represent a molecular mechanism by which this compound acts as an antitumor agent in colon carcinogenesis.

Leung et al., (2008) examine the relationship between the lipid-soluble antioxidant vitamins, the extent of free radical activity, tumor stage, the systemic inflammatory response and survival in patients with colorectal cancer. In this study showed that the systemic inflammatory response was associated with a reduction of lipid-soluble antioxidant vitamins, whereas advanced tumor stage was associated with increased lipid peroxidation in patients with colorectal cancer. Of the antioxidant vitamins measured, only retinol was independently associated with cancer-specific survival.

Cervical Intraepithelial Neoplasia

Given the promise of diet and micronutrient supplementation on cancer chemoprevention in numerous epidemiological trials, four randomized clinical trials using β-carotene as a possible chemopreventative agent for cervical cancer have been completed to date with mixed results (Fairleyet al., 1996).

One report presents data from a 2-year, randomized, placebo-controlled, chemoprevention trial evaluating the effect of β-carotene in the treatment of high-grade CIN (CIN 2 and 3). Intermediate biomarkers of cervical cancer risk, including grade of CIN (cervical intraepithelial neoplasia) and HPV (Human papillomavirus) presence, and risk category (high, intermediate, low, indeterminate, or none) were also evaluated. Serum and vaginal micronutrient levels were measured to determine whether they were predictive of lesion regression. In conclusion, cervical biopsies and the associated cell-mediated immune

response may be partly responsible for the high rate of regression of high-grade lesions and the low rate of detection of HPV in the initial cervical scrapings (Keefe et al., 2001).

Case-control studies exploring the relationship between diet and cervical dysplasia have demonstrated that a low intake of vitamins A, C, and β-carotene was associated with an increased risk of cervical dysplasia (Romney et al., 1981). Measurement of plasma levels of various micronutrients has supported this observation, with low levels typically associated with an increased risk of CIN (cervical intraepithelial neoplasia) and cervical cancer.

Palan et al. (1988) reported lower levels of vitamin C and β-carotene in the plasma of women with cervical dysplasia than in normal controls. A similar finding was reported by Batieha et al. (1993) in a nested case control study in which 50 women who developed cervical cancer or carcinoma-*in situ* over a 15-year period had significantly lower prediagnostic serum β-carotene levels than 99 matched controls.

Lung Cancer

Although cigarette smoking has decreased in some countries, there are still about 1,200 million smokers in the world. China alone has approximately 300 million male smokers, about the same as the population of the United States. Globally, about 57% of men and 10% of women smoke tobacco products. Cigarettes are the main type of tobacco product worldwide. About 5.5 trillion cigarettes were consumed annually in 1990-2000, about 1,000 cigarettes for every person on Earth. Over 15 billion cigarettes are smoked per day (Mackay and Eriksen, 2002).

The association between smoking, cancer and/or coronary artery disease is universally accepted. Cigarette smoking causes well over 1 million cancer deaths annually in the world and about 30% of all cancer deaths in developed countries. Concomitantly, smoking has been reported to increase atherosclerotic diseases by about 50% and at least doubles the incidence of coronary hearth disease, by inducing endothelial dysfunction, oxidation of LDL cholesterol, higher levels of adhesion molecules and fibrinogen, increased platelet aggregation, higher prevalence of vascular spasm and by reducing HDL cholesterol concentration (Genest et al., 2001).

At least 4700 constituents of mainstream cigarette smoke have been identified. Among them, 62 compounds are carcinogenic in laboratory animals, and 15 are carcinogenic in humans. They include: polycyclic aromatic hydrocarbons (PAH}, heterocyclic compounds, N-nitrosamines, aromatic amines, heterocyclic aromatic amines, several aldehydes, phenolic compounds, volatile hydrocarbons, nitrohydrocarbons, several organic compounds, some metals and inorganic compounds (Munteanu and Didilescu, 2007). Moreover, radioactivity in tobacco smoke may strongly contribute to its carcinogenicity together with another 600 additives which are used in the technological process.

Moreover, cigarette smoke contains high concentrations of two different populations of free radicals, one in the tar component and the other in the gas component phase of smoke (Rustemeier et al., 2002). While the strong oxidants in gas phase smoke can rapidly initiate DNA, lipid and protein oxidation, the polyphenol-quinone redox couples in tar can more

slowly generate radicals over a sustained period. Moreover, fine particles contained in smoke can induce plaque deposits in arteries, causing vascular inflammation and atherosclerosis.

Whereas a causal relation between cigarette smoking and lung cancer was firmly established > 4 decades ago, not until the 1970s did the potential role of dietary factors in the development of lung cancer garner widespread interest. This was spurred by the pioneering work of Bjelke (1975) and Kvale et al (1983) exploring the potential protective role of vitamin A and by a seminal review by Peto et al (Peto et al., 1981) discussing the chemopreventive potential of β-carotene, a provitamin A carotenoid and tended to support an inverse association of lung cancer incidence with β-carotene intake and with serum concentrations of β-carotene. This evidence led to the initiation of several large scale randomized chemoprevention trials to test the hypothesis that β-carotene supplements protected against lung cancer, but those trials had disappointing results. Indeed, β-carotene supplementation actually was found to increase the risk of lung cancer in high-risk populations (Omenn et al., 1996a, Virtamo et al., 2003).Whereas high-dose β-carotene supplementation is ineffective in reducing lung cancer risk in randomized trials, many questions remain about the potential benefits of the intake of lower doses of β-carotene over prolonged periods. Furthermore, there is substantial interest in the potential role of other carotenoids in lung cancer prevention.

Evidence from observational epidemiologic studies rapidly accumulated epidemiological studies have provided evidence for an association between high β-carotene uptake, or a high β-carotene plasma concentration and reduced risks for cancer, especially lung cancer. In addition, animal studies demonstrated anticarcinogenic activity for β-carotene. Unexpectedly, in three large clinical intervention trials, β-carotene supplementation either showed no effect, or was associated with an increased incidence of lung cancer (ATBC; CARET) (IARC, 1998). The mechanism(s) by which β-carotene may be associated with an increased risk of lung cancer in heavy smokers is, as yet, unknown.

As part of efforts to address this deficiency, Goralczyka et al., (2005) investigated the influence of β-carotene intake on tobacco smoke carcinogen-induced lung tumorigenesis. With the AJJ strain of mice, we chose a model that is widely used in the search for chemopreventive agents (Stoner, 1998). The model is sensitive to all known human carcinogens and most suspected human carcinogens, such as the tobacco-specific carcinogen 4-(N-Methyl-N- nitrosamino)-1-(3-pyridyl)-1-butanone) (NNK) (Hecht, 2002). Found no effect of β-carotene, irrespective of dose and time point of treatment, on the tumor formation in the NNK-initiated A/J-mouse lung cancer model. The enhancement of NNK-induced bronchial epithelial cell proliferation by β-carotene shortly after initiation is unlikely to be predictive for later tumor formation. The modulation of RA-responsive gene expression levels by NNK and/or β-carotene was not predictive for later tumor development. Moderate increases in RARβ by β-carotene alone are indicative of intact β-carotene metabolism and sensitivity to RA in the mice.

In another study ferrets were given a high-dose β-carotene supplement equivalent to 30 mg per day in humans, and exposed cigarette smoke or both for six months. A strong proliferative response in lung tissue and squamous metaplasia were observed in all β-carotene-supplemented animals, and this response was enhanced by exposure to tobacco smoke. Had statistically significantly lower concentrations of retinoic acid in lung tissue, and

they exhibited reductions in RAR-β gene expression (a tumor suppressor gene). Further, ferrets given a high-dose beta-carotene supplement and exposed to tobacco smoke had fourfold elevated expressions of c-jun and c-fos genes (Russell, 2002).

Previous reviews of this topic (Ruano-Ravina et al., 2006; Ziegler et al., 1996; Cooper et al., 1999; Handelman, 2001) were not performed systematically. Given the public health importance of clarifying the potential role of carotenoids in lung carcinogenesis and given the extensive and diverse body of evidence available, Gallicchio et al., (2008) conducted a systematic and quantitative review of the evidence, derived from randomized clinical trials (RCTs) and from prospective observational studies, for the associations between carotenoids and the risk of lung cancer. Six randomized clinical trials examining the efficacy of β-carotene supplements and 25 prospective observational studies assessing the associations between carotenoids and lung cancer were analyzed by using random-effects meta-analysis. The pooled relative risk (RR) for the studies comparing β-carotene supplements with placebo. Among the observational studies that adjusted for smoking, the pooled RRs comparing highest and lowest categories of total carotenoid intake and of total carotenoid serum concentrations.

β-Carotene supplementation is not associated with a decrease in the risk of developing lung cancer. Findings from prospective cohort studies suggest inverse associations between carotenoids and lung cancer; however, the decreases in risk are generally small and not statistically significant. These inverse associations may be the result of carotenoid measurements' function as a marker of a healthier lifestyle (higher fruit and vegetable consumption) or of residual confounding by smoking (Shekelle et al., 1981).

Because approximately one-sixth of Americans regularly consume multivitamins, it is relevant to understand whether and how much beta-carotene exists in national brand multivitamins. Although a smaller proportion of smokers consume multivitamins than nonsmokers, some adopt the habit of taking multivitamins as part of a healthier lifestyle. Patients with a history of lung cancer, who often have a significant smoking history, as well as their family members, are also more likely to take dietary supplement (Pisani et al., 1986).

An important observation has emerged from these chemoprevention trials. Some studies have identified beta-carotene as being associated with an increased risk of lung cancer, especially among participants who are active smokers or have a significant smoking history. A previous meta-analysis containing data from three large trials has suggested a marginally increased risk of lung cancer associated with beta-carotene supplementation among current smokers or former smokers (Touvier et al., 2005).

Tanvetyanon and Bepler (2008), systematically reviewed published randomized controlled trials that reported on the effect of beta-carotene on the incidence of lung cancer. To understand the differential effect of β-carotene in the high-risk populations who are current smokers or former smokers, performed an meta-analyses in both subgroups separately. In addition, evaluated the beta-carotene content of a national brand multivitamin sample.

Some studies have suggested that beta-carotene supplementation may increase the risk of lung cancer, particularly among smokers or former smokers. β-Carotene, a provitamin A, is available in multivitamins. In the current study, the authors investigated the risk of lung cancer associated with beta-carotene in smokers or former smokers and surveyed the beta-

carotene content in national brand multivitamins. Four studies contributing 109,394 subjects were available for analysis. The average daily beta-carotene dosage in these trials ranged from 20 to 30 mg daily. High-dose β-carotene supplementation appears to increase the risk of lung cancer among current smokers. Although beta-carotene was prevalent in multivitamins, high-dose β-carotene was observed among multivitamin formulas sold to promote visual health (Tanvetyanon and Bepler, 2008).

Gastrointestinal Cancer

The incidence of esophageal adenocarcinoma has been increasing rapidly among many countries. Antioxidant intake is a potentially modifiable protective factor, although the results from individual studies are inconclusive. In an study, were evaluated the associations between vitamin C, vitamin E, or β-carotene/vitamin A and the risk of esophageal adenocarcinoma or the adjacent gastric cardia (gastroesophageal junction) adenocarcinoma. Studies (1 cohort, 9 case-control; 1,057 esophageal and 644 cardia cases). Higher intakes of vitamin C, β-carotene/vitamin A, and vitamin E were inversely associated with the risk of esophageal adenocarcinoma. β-Carotene intake was also inversely associated with the risk of cardia adenocarcinoma. Pooled results from observational studies suggest that antioxidant intake may be protective against esophageal adenocarcinoma; the data do not support a consistent association between antioxidant intake and the risk of cardiac carcinoma. These findings suggest possible etiological differences between these two adjacent malignancies (Kubo and Corley, 2007).

Oxidative stress may cause gastrointestinal cancers. Epidemiologic studies of vitamin A, retinol (preformed vitamin A), and provitamin A carotenoids in relation to the risk of gastric cancer have documented inconsistent results. The evidence on whether antioxidant supplements are effective in preventing gastrointestinal cancers is contradictory. Results supported not find evidence that the studied antioxidant supplements prevent gastrointestinal cancers. On the contrary, they seem to increase overall mortality (Bjelakovic et al., 2008).

Mechanisms of β-Carotene in Cancer

Although several mechanisms have been proposed to explain the putative role of β-carotene in cancer, no studies have investigated a possible influence of β-carotene on caveolin-1 (cav-1) pathway, an important intracellular signalling deregulated in cancer. Here, different human colon and prostate cancer cell lines, expressing (HCT-116, PC-3 cells) or not (Caco-2, LNCaP cells) cav-1, were treated with varying concentrations of β-carotene (0.5-30 muM) for different periods of time (3-72 h) and the effects on cell growth were investigated. The results of this study show that: a) β-carotene acted as a growth-inhibitory agent in cav-1-positive cells, but not in cav-1-negative cells; b) in cav-1-positive cells, the carotenoid down-regulated in a dose- and time-dependent manner the expression of cav-1 protein and mRNA levels and inhibited AKT phosphorylation which, in turn, stimulated apoptosis by increasing the expression of β-

catenin and c-myc and the activity of caspases-3, -7, -8, -9; when the carotenoid was removed from culture medium, a progressive increase in cell growth was observed with respect to β-carotene-treated cells; c) the transfection of cav-1 in cav-1-negative cells increased cell sensitivity to β-carotene, by inducing apoptosis. This effect was accompanied by a reduction of both cav-1 and AKT phosphorylation and by an increase of c-myc and beta-catenin expression. Silencing of c-Myc attenuated β-carotene-induced apoptosis and β-catenin expression. All together, these data suggest that the modulation of cav-1 pathway by β-carotene could be a novel mechanism by which the carotenoid acts as a potent growth-inhibitory agent in cancer cells (Palozza et al., 2008).

Several mechanisms have been proposed by which carotenoids may modulate cellular response to smoke (Palozza et al., 2008a). Carotenoids have been reported to modulate:

- The levels of smoke-related reactive oxygen species (ROS) by acting as redox agents.
- Phase I carcinogen-bioactivating enzymes, including activators of cigarettes smoke carcinogens, such as polycyclic aromatic hydrocarbons
- Carcinogens binding to DNA.
- Molecular pathways, involved in cell proliferation and apoptosis affected by smoke, including retinoic acid signalling.

Clinical Studies on Cancer

Photooxidative stress may play a role in the etiology of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), collectively termed nonmelanoma skin cancer (NMSC). Antioxidant vitamins as β-carotene might therefore offer some protection (Steenvoorden and van Henegouwen, 1997).

A nested case-control study was conducted within the Physicians' Health Study, a randomized, double-blind, placebo-controlled trial of 50 mg β-carotene supplementation on alternate days with 12 years of follow-up. Study subjects were 1,338 men ages 40 to 84 years at baseline who, during follow-up, developed a NMSC, (including 1,156 with BCC and 166 with SCC) and an age and smoking–matched control group of 1,338 men who remained free of NMSC at the time of diagnosis of the case. This study provides substantial evidence that there is no beneficial effect of 12 years of β-carotene supplementation on the risk of NMSC, including BCC and SCC, among subjects with the lowest baseline plasma levels of β-carotene, α-tocopherol, or vitamin A. Furthermore, there is no association between plasma levels of β-carotene, α-tocopherol, or vitamin A and risk of NMSC. Until further evidence emerges, risk reduction through limiting exposure to UV light remains the best available strategy for prevention of NMSC (Schaumberg et al., 2004).

In addition, randomised trials (211,818 participants), comparing antioxidant supplements (β-carotene, α-tocopherol, or vitamin A) to placebo/no intervention examining occurrence of gastrointestinal cancers. Not find convincing evidence that antioxidant supplements prevent

gastrointestinal cancers. On the contrary, antioxidant supplements seem to increase overall mortality (Bjelakovic et al., 2008).

However, Larsson et al., (2007) reported that high intakes of vitamin A and retinol from foods only (dietary intake) and from foods and supplements combined (total intake) and of dietary α-carotene and β-carotene were associated with a lower risk of gastric cancer in a study cohort consisted of 82, 002 Swedish adults aged 45-83.

Randomised trials testing β-carotene supplementation, alone or in combination with other supplements, have not supported lower cancer rates (Greenberg et al, 1990; Blot et al, 1993; Li et al, 1993). Of seven trials, performed on α-Tocopherol and β-Carotene the Cancer Prevention Study Group in 1994, one observed a significant benefit on cancer mortality (Blot et al, 1993), four reported no significant benefit or harm on the incidence of cancer and cardiovascular disease (Greenberg et al, 1990; Li et al, 1993; Hennekens et al, 1996; Lee et al, 1999), while the remaining two trials found an unexpected, but significant increase in lung cancer incidence (Omenn et al, 1996a; The α-tocopherol, β-carotene Cancer Prevention Study Group, 1994). The only trial reporting a benefit of β-carotene supplementation tested a combination of β-carotene, vitamin E, and selenium among poorly nourished adults in China (Blot et al, 1993). This has raised the hypothesis that any benefit of β-carotene supplementation may be limited to those with low levels of plasma β-carotene (Lee et al, 1999).

In another study contributing 109,394 subjects with average daily β-carotene dosage in these trials ranged from 20 to 30 mg daily. Among current smokers, β-carotene supplementation was found to be significantly associated with an increased risk of lung cancer. Among former smokers, there was no significant increase noted. In a sample of 47 common multivitamins, β-carotene was present in 70% of the identified formulas. The median dosage of β-carotene was 0.3 mg (range, 0-17.2 mg) daily. The β-carotene content was found to be significantly higher among multivitamins sold to improve visual health than among other multivitamins, with a median daily dosage of 3 mg (range, 0-24 mg).High-dose β-carotene supplementation appears to increase the risk of lung cancer among current smokers. Although β-carotene was prevalent in multivitamins, high-dose β-carotene was observed among multivitamin formulas sold to promote visual health (Tanvetyanon and Bepler, 2008).

In another trial conducted for a 5-8 years (median, 6.1 years) period in a total of 29,133 men aged 50-69 years who smoked five or more cigarettes daily were randomly assigned to receive α-tocopherol (50 mg), β-carotene (20 mg), or a placebo daily. Data regarding smoking and other risk factors for lung cancer and dietary factors were obtained at study entry, along with measurements of serum levels of α-tocopherol and β-carotene. Incident cases of lung cancer (n = 894) were identified through the Finnish Cancer Registry and death certificates. Each lung cancer diagnosis was independently confirmed, and histology or cytology was available for 94% of the cases. Intervention effects were evaluated by use of survival analysis and proportional hazards models. Supplementation with α-tocopherol or β-carotene does not prevent lung cancer in older men who smoke. β-Carotene supplementation at pharmacologic levels may modestly increase lung cancer incidence in cigarette smokers, and this effect may be associated with heavier smoking and higher alcohol intake. While the

most direct way to reduce lung cancer risk is not to smoke tobacco, smokers should avoid high-dose β-carotene supplementation (Albanes et al., 1996).

It is also reported in other trial that was carried on the β-Carotene and retinol efficacy (CARET) tested the combination of 30 mg β-carotene and 25 000 IU retinyl palmitate (vitamin A) taken daily against placebo in 18,314 men and women at high risk of developing lung cancer. The CARET intervention was stopped 21 months early because of clear evidence of no benefit and substantial evidence of possible harm; there were 28% more lung cancers and 17% more deaths in the active intervention group (active = the daily combination of 30 mg β-carotene and 25 000 IU retinyl palmitate), (Omenn et al., 1996a). Results showed that CARET participants receiving the combination of β-carotene and vitamin A had no chemopreventive benefit and had excess lung cancer incidence and mortality. These results are highly consistent with those found for β-carotene and α-tocopherol by Cancer Prevention Study in 29,133 male smokers in Finland, performed a randomized double-blind. In this trial found no reduction in the incidence of lung cancer among male smokers after five to eight years of dietary supplementation with α-tocopherol or β-carotene. In fact this trial raises the possibility that these supplements may actually have harmful as well as beneficial effects (Heinone and Albanes,1994).

In another study, was observed the effect of α-tocopherol, β-carotene Cancer Prevention Study (ATBC Study), whose participants were randomly assigned to four supplementation groups: (a) α-tocopherol (AT), 50 mg/day; (b) β-carotene (BC), 20 mg/day; (c) both AT and BC; and (d) placebo. Included 15,538 ATBC participants who had been randomized within the areas of three major cities in southern Finland. Cases of colorectal adenoma (n = 146) were identified by the pathology laboratories in the study areas, and these participants' medical records were collected and reviewed. α-Tocopherol supplementation increased the risk for, whereas β-carotene supplementation had no effect on the risk. Slightly more prediagnosis rectal bleeding and intestinal pain occurred in those adenoma cases who received α-tocopherol supplements than in those who did not. Thus, some bias may have resulted, with α-tocopherol supplementation leading to more colonoscopies and, thus, to an increased detection of incident polyps in this group (Malila et al., 1999).

In addition, in other trial was studied the effect of β-carotene supplementation on colorectal adenoma recurrence among subjects in a multicenter double-blind, placebo-controlled clinical trial of antioxidants for the prevention of colorectal adenomas. A total of 864 subjects who had had an adenoma removed and were polyp-free were randomly assigned (in a factorial design) to receive β-carotene (25 mg or placebo) and/or vitamins C and E in combination (1000 mg and 400 mg, respectively, or placebo), and were followed with colonoscopy for adenoma recurrence 1 year and 4 years after the qualifying endoscopy. A total of 707 subjects had two followup examinations and provided smoking and alcohol use data (Baron et al., 2003). The results obtained showed that among subjects who neither smoked cigarettes nor drank alcohol, β-carotene was associated with a marked decrease in the risk of one or more recurrent adenomas but β-carotene supplementation conferred a modest increase in the risk of recurrence among those who smoked. For participants who smoked cigarettes and also drank more than one alcoholic drink per day, β-carotene doubled the risk of adenoma recurrence. Alcohol intake and cigarette smoking appear to modify the effect of β-carotene supplementation on the risk of colorectal adenoma recurrence (Baron et al., 2003).

Recently, was observed in a case-control study of lung cancer in Hawaii, a negative association with risk for several vegetables rich in specific carotenoids (some of which contain little β-carotene) similar to that found for an index of β-carotene intake (Le Marchand et al., 1989). Thus, the data were suggestive of a protective effect against lung cancer for carotenoids other than β-carotene. Were also found an inverse association with total intake of vegetables which was stronger than that for β-carotene or particular carotenoid-rich food groups, suggesting that different constituents of vegetables may interact additively (or synergistically) to protect against lung cancer (Le Marchand et al., 1989). In these data, also found no association with vitamin C, fiber, or fruits.

Since the publication of this report, food composition values have become available for the main carotenoids. Reexamined the data using new carotenoid values to more directly assess the associations of dietary β-carotene, α-carotene, lutein, lycopene, and β-cryptoxanthin with lung cancer. The analysis included interviews with 230 men and 102 women with lung cancer and 597 men and 268 women as controls, frequency-matched to the patients by age and sex. A previously validated quantitative diet history assessed the usual intake of foods rich in carotenoids. After adjusting for smoking and other covariates, no association was found with lung cancer risk for dietary lycopene or β-cryptoxanthin intake, whereas dose-dependent inverse associations of comparable magnitude were found for dietary β-carotene, α-carotene, and lutein (Le Marchand et al., 1993).When subjects were cross-classified by their joint intakes of the latter three carotenoids, those who had a high intake (> median) for all three had the lowest risk for lung cancer. In a similar two-way interaction analysis, the previously reported inverse association of lung cancer with vegetable consumption in these data was found to be stronger than that with intake of these three carotenoids. This analysis provides further evidence for a protective effect of certain carotenoids against lung cancer and for the greater protection afforded by consuming a variety of vegetables compared to only foods rich in a particular carotenoid.

Until recently, only α-carotene had been examined in dietary epidemiological studies, most often using an index based on vitamin A intake from plant sources. These studies have been very consistent in suggesting a protective effect for β-carotene intake, especially against epithelial cancers of the respiratory tract (Khachik et al., 1991). Studies measuring serum β-carotene levels have also been supportive. Although other carotenoids are found in the diet and serum of western populations at levels similar to those of β-carotene, they have rarely been studied in relation to cancer risk

It was also reported in another study in various areas of Italy between 1992 and 2006 on 454 women with incident, histologically confirmed endometrial cancer and 908 controls admitted to the same network of hospitals of cases for acute, non-neoplastic conditions. Intake of carotenoids and retinol was computed from a validated and reproducible food frequency questionnaire. Comparing the highest to the lowest quartile of intake, the ORs of endometrial cancer were 0.69 for β-carotene, 0.65 for β-cryptoxanthin, and 0.59 for lutein plus zeaxanthin intake. No association emerged with retinol, α-carotene, and lycopene. The results support a favorable role of selected dietary carotenoids on endometrial cancer risk (Pelucchi et al., 2008).

The effect of the diet was compared in 450 lung cancer cases (296 males, 154 females) with those of 902 controls (587 males, 315 females). Cases were lung cancer patients

diagnosed between August 1980 and July 1984 in three western New York counties, while controls were selected from the general population of these same counties. Usual diet was estimated by detailed interviews using a modified food frequency method. Case-control comparisons were made for dietary fat, protein, fiber, calories, cholesterol, and vitamins A, C, and E according to quartiles of intake, adjusting for age and pack-years of cigarettes by multiple logistic regression. Risk was lower for males in the lowest quartile of total dietary fat intake compared with those in the highest quartile, although the overall trend in the association with dietary fat was not statistically significant. Likewise, there was a weak, but not statistically significant, direct association between dietary cholesterol and lung cancer in men. The intake of carotene from fruits and vegetables was much more strongly associated with reduced cancer risk. For males, the relative risks by quartiles was considerably weaker, and was not statistically significant. These findings are generally in agreement with those of several previous studies. The risk reduction associated with vitamin A from fruits and vegetables (carotene) was most evident for males, for those with squamous cell cancers, for light or ex-smokers, and for those over 60 years of age (Byers et al., 1987).

An inverse association between β-carotene intake and risk of neoplasms has been described largely in observational studies, thus leading researchers to design many intervention studies with this antioxidant (Hercberg et al., 2004). However, its safety is debated (Greenwald, 2003), as some intervention studies have suggested a positive association of high doses of supplemental β-carotene, especially in smokers, with lung cancer (Omenn et al., 1996; Albanes et al., 1996) and with digestive cancers, during the trial or the post-trial follow-up (Malila et al., 2002). A meta-analysis of intervention studies on digestive tract cancers suggested a direct association between cancer incidence and intake of β-carotene alone or combined with retinol or tocopherol (Bjelakovic et al., 2004). In an intervention study of patients with colorectal adenomas, a precancerous lesion for colorectal cancer, an inverse association between adenoma recurrence and β-carotene intake was observed in non smokers, but a direct association was observed in those smokers who drank at least one alcoholic drink per day (Baron et al., 2003). In contrast, a pooled analysis of seven cohorts (Mannisto et al., 2004) and two intervention studies (Lee et al., 1999; Hennekens et al., 1996) did not show a statistically significant interaction between β-carotene and smoking with cancer incidence.

A potential interaction between β-carotene intake and smoking on the risk of tobacco-related cancers was investigated in 59,910 women participating in the French Etude Epidemiologique de Femmes de la Mutuelle Generale de I'Education Nationale. After a median follow-up period of 7.4 years, 700 women had developed cancers known to be associated with smoking (e.g., lung, head, and neck, urinary tract, digestive tract, cervix, thyroid, and ovary). Among women who had never smoked, there was a significant inverse association between β-carotene intake from both diet and supplements and the risk of all smoking-related cancers. Supplement users had a 56% lower risk of developing such cancers, compared with women in the lowest tertile of β-carotene intake. In contrast, among women who had ever smoked (including current and former smokers), increasing β-carotene intake was associated with an increase in the incidence of smoking-related cancers. Smokers who took β-carotene supplements had more than twice the risk of such cancers as did women in the lowest tertile of β-carotene intake (hazard ratio = 2.14; 95%) (Touvier, 2005). The results

were consistent with the findings of previous studies that have reported a positive association between β-carotene intake and risk of some neoplasms in smokers (Omenn et al., 1996; Albanes et al., 1996) and with those that observed an interaction between β-carotene intake and smoking on the risk of some cancers or precancerous lesions (Baron et al., 2003) . Indeed, long-term follow-up of the β-carotene and retinol efficacy trial suggests a stronger association between β-carotene intake and risk of tobacco-related cancer in women than in men (Goodman et al., 2004), which, added to a higher supplement use (Knudsen et al., 2002), may therefore represent a problem in view of the increasing exposure of women to tobacco.

In another study, it was observed that increased lung cancer risk was associated with low vegetable and fruit intake in current and recent cigarette smokers and in pipe and/or cigar users. Risk was not elevated in cigarette smokers who had quit more than 5 years earlier or in never smokers. The effects of β-carotene intake and of α-carotene intake on lung cancer risk were similarly modified by smoking history. Thus, as before, the importance of diet was explored in current (464 case patients and 177 control subjects) and recent (59 case patients and 31 control subjects) smokers. Current and recent smokers in the lowest quartile of α-carotene intake had a smoking-adjusted risk more than twice that of smokers in the highest quartile of intake, whereas the corresponding risks associated with intakes of β-carotene and of lutein/zeaxanthin were increased only about 60% (Ziegler et al., 1996a).

It is also reported that in a group of 258 lung cancer cases and 515 controls, serum/plasma concentrations were significantly lower among cases than controls for cryptoxanthin, β-carotene, and lutein/zeaxanthin. Modest nonsignificant case-control differences in a protective direction were noted for α-carotene and ascorbic acid. There were only trivial differences for lycopene, α-tocopherol, selenium, and peroxyl radical absorption capacity. Findings are reported for males and females and for persons who had never smoked cigarettes, former smokers, and current smokers at baseline. These results and those from previous studies suggest that β-carotene is a marker for some protective factor(s) against lung cancer; that cryptoxanthin, α-carotene, and ascorbic acid need to be investigated further as potentially protective factors or associates of a protective factor; and that lycopene, α-tocopherol, selenium, and peroxyl radical absorption capacity are unlikely to be associated with lung cancer risk. Until specific preventive factors are identified, the best protection against lung cancer is still the avoidance of airborne carcinogens, especially tobacco smoke; second hest is the consumption of a diet rich in fruits and vegetables (Comstock et al., 2008).

In 1957, 3,102 men (aged 40-55 years) were randomly selected for the Western Electric Study. The effects of β-carotene and retinol intake on lung cancer risk during 19 years no showed significant differences in mean intake other nutrients. Also this study showed no association between the level of serum cholesterol and the intake of retinol and β-carotene (Shekelle et al., 1981).

Discussion

In recent decades the presence of carotenoids in our food supply and their role in human health have been of unprecedented interest. Some carotenoids are vitamin A precursors and about a dozen carotenoids are found in human plasma, depending on diets rich in fruits and

vegetables. Fifty carotenoids are typically present in the human diet and several are found in human plasma, including β-carotene, α-carotene, lycopene, zeaxanthin and lutein. Carotenoids are potent antioxidants and are known to affect many different cellular pathways (Krinsky et al., 2002). Moreover, several observational and prospective epidemiological studies have consistently shown an inverse relationship between dietary intakes or blood levels of β-carotene, one of the most known carotenoids, and cardiovascular diseases (Honarbakhsh and Schachter, 2008) or several types of cancer (Gerster, 1995).

On the other hand, there is some contradictory evidence from human intervention trials using β-carotene supplements. An increase in the risk of lung cancer among smokers who took β-carotene supplements was reported in the alpha-tocopherol, beta-carotene Cancer Prevention Trial (ATBC) in Finland (The α-tocopherol, β-carotene Cancer Prevention Study Group, 1994) and among smokers and asbestos workers in the β-carotene and retinol efficacy trial (CARET) in USA (Hennekens et al., 1996), but not among healthy male physicians in the Physicians' Health Study in USA (Omenn et al.,1996). Moreover, most of the clinical studies for primary or secondary prevention of atherosclerosis failed to show a protective effect or even showed adverse effects after administration of β-carotene (Siekmeier et al., 2006). These findings aroused widespread scientific debate and raised the suspicion that carotenoids may even have dangerous effects in human body under certain circumstances.

In 1981 it was hypothesized that a high dietary intake of β-carotene might reduce human cancer rates. Since then, several observational epidemiologic studies have addressed this topic. The results of both case-control and cohort studies show a remarkable consistency for the association of increased lung cancer risk with low amounts of dietary β-carotene or low plasma β-carotene concentrations. For stomach cancer, the evidence is also consistent, although the number of studies is more modest. For breast and prostate cancer, the studies indicate no consistent association of plasma or dietary β-carotene and reduced cancer risk. For colorectal cancer, the effect will be moderate, if existent. However, overall results are promising and several plausible cancer preventive mechanisms have been reported for β-carotene (van Poppel et al., 1995).

Prospective and retrospective studies suggest that carotenoids may reduce the risk of certain other cancers; however, too few studies have looked at these sites to examine the consistency of the evidence. Although clinical trials of the efficacy of β -carotene in cancer prevention are underway, it is still necessary and prudent to continue well-designed prospective and retrospective studies of the carotenoid hypothesis. However, the importance of other carotenoids, other constituents of vegetables and fruits, and other nutrients whose levels in the blood are partially correlated with those of β-carotene has not been adequately explored.

Although initial studies suggested that persons with lower levels of serum retinol have higher future rates of lung cancer, this idea was not confirmed in subsequent investigations. Prediagnostic levels of β-carotene in blood, however, have been inversely related with risk of lung cancer. Available data thus strongly support the hypothesis that dietary carotenoids reduce the risk of lung cancer, but the data are also compatible with the possibility that some other factor in these foods is responsible for the lower risk. Even if ultimately shown to be causal, the relation between diet and lung cancer is modest compared with the deleterious effect of cigarette smoking.

Although initial studies suggested that persons with lower levels of serum retinol have higher future rates of lung cancer, this idea was not confirmed in subsequent investigations. Prediagnostic levels of β-carotene in blood, however, have been inversely related with risk of lung cancer. Even if ultimately shown to be causal, the relation between diet and lung cancer is modest compared with the deleterious effect of cigarette smoking.

In a supplementation study, high β-carotene intake was associated with a decrease in DNA adduct levels in nonsmokers but with an increase in such adducts in smokers (Welch et al., 1999). Suggested mechanisms for this effect are complex and debated (Lotan, 1999). In *in vitro* models, β-carotene may serve as an antioxidant or as a prooxidant, depending on the redox potential of the biologic environment in which it acts, as reviewed previously (Palozza et al., 2003). Although β-carotene exerts a growth inhibitory and proapoptotic effect on malignant colonic cell lines (Palozza et al., 2005) , it also enhances DNA oxidative damage and modifies p53-related pathways of cell proliferation and apoptosis when cells are exposed to tobacco smoke condensate (Palozza et al., 2004) .

Although β-carotene may act as a cocarcinogen, there is no evidence that smokers should avoid consuming β-carotene rich foods such as fruit and vegetables, in which other components, such as vitamins C and E, may counteract a potentially deleterious interaction of β-carotene with smoking. In a study, former smokers were more likely to take supplements than current or never smokers, as reported elsewhere. This behavior, which may have been part of a healthier lifestyle for women who decided to stop smoking, may have unexpected adverse effects when supplements include β-carotene. Not smoking and consuming relatively high doses of β-carotene were associated with the lowest level of risk of tobacco-related cancer, in agreement with ongoing public health advice (Paolini et al., 2003).

In conclusion, the interaction between tobacco and β-carotene, which was initially described for lung cancer (Albanes et al., 1996), may extend to other tobacco-related cancers. In our cohort, tobacco-related cancers represented 23.0% of all cancers observed during the study period. This rate is slightly lower than the 30% reported in the literature (Stein and Colditz, 2004) but consistent with the study population's relatively low exposure to tobacco. This proportion emphasizes the public health importance of our results. Because the observed interaction between β-carotene and smoking on tobacco-related cancer risk could strongly influence a global effect of β-carotene on risk of neoplasms, future studies on the effect of this nutrient should include stratification by smoking status. In general, studies should systematically investigate potential interactions between nutrients and environmental or genetic factors (Palli et al., 2004).

The generally accepted causes of lung cancer are inhalant: tobacco smoke; dusts or fumes containing carcinogen, such as arsenic, asbestos, chloromethyl ether and chromates; and gases such as radon (Peto et al., 1981). However, the fact that not all persons exposed to even high concentrations of these airborne pollutants develop cancer suggests that there are substances that can prevent or inhibit carcinogenesis. An appealing hypothesis involves the following simplified chain of events. Many carcinogens create free oxidative radicals that damage cells; damaged cells are prone to develop malignant changes; and antioxidants can neutralize free radicals, thereby preventing cell damage and the subsequent development of cancer (Block, 1992). This hypothesis would be strengthened if it could be consistently demonstrated that persons who developed cancer had lower concentrations of antioxidant

.Substances in their blood before they developed cancer than persons who had remained free of cancer. An impressive number of observational studies have addressed this hypothesis with respect to the association of serum or plasma concentrations of several antioxidants and lung cancer (Comstock and Helzlsouer, 1997). The antioxidants assayed in these studies include retinol, total carotenoids, β-carotene, α-tocopherol and selenium, the last being a surrogate for the selenium-containing enzyme, glutathione peroxidase. Serum retinol showed only a trivial and inconsistent association with the subsequent development of lung cancer. The results of the two studies that reported on total carotenoids were inconsistent. A larger number of studies dealing with α-tocopherol showed only weak associations; they were also inconsistent in the direction of the associations. The results of studies involving serum or toenail selenium were stronger but were also inconsistent. In contrast, the associations of β-carotene concentrations in the serum were remarkably consistent in showing a considerably lower risk among persons with higher serum concentrations (Comstock et al., 2008).

The β-carotene-lung cancer association is sufficient to affect recommendations only insofar as they support current guidelines concerning enhanced vegetable and fruit consumption. It is clear that persons who eat a relatively large quantity of vegetables and fruit have a substantially lower risk of developing lung cancer (Steinmetz and, Potter, 1991; Block et al., 1992a) and they may experience less cardiovascular disease and delayed mortality as well. Although many available studies (van Poppel and Goldbohm, 1995; Ziegler et al., 1996b) strongly implicate ß-carotene and possibly other carotenoids as among the putative agents of benefit, certainty around this issue is lacking. Protective associations for greater consumption of vegetables and fruit have often been stronger than those for β-carotene or total carotenoid intake specifically, suggesting the possibility of an etiologic relation with lung cancer for something in such diets beyond one or a few of the micronutrients that is, the whole being greater than the sum of its parts. Further, the supplementation trials suggest not only lack of benefit of β-carotene in lung cancer prevention, but possible harm in smokers from not only lung cancer but overall mortality as well.

CVD is a major cause of mortality and morbidity in the Western world. In recent years its importance has expanded internationally and it is believed that by 2020 it will be the biggest cause of mortality in the world, emphasising the importance to prevent or minimise this increase. A beneficial role for vitamins in CVD has long been explored but the data are still inconsistent. While being supported by observational studies, randomised controlled trials have not yet supported a role for vitamins in primary or secondary prevention of CVD and have in some cases even indicated increased mortality in those with pre-existing late-stage atherosclerosis. The superiority of combination therapy over single supplementation has been suggested but this has not been confirmed in trials. Studies have indicated that β-carotene mediates pro-oxidant effects and it has been suggested that its negative effects may diminish the beneficial effects mediated by the other vitamins in the supplementation cocktail. The trials that used a combination of vitamins that include β-carotene have been disappointing. However, vitamin E and vitamin C have in combination shown long-term anti-atherogenic effects but their combined effect on clinical endpoints has been inconsistent. Studies also suggest that vitamins would be beneficial to individuals who are antioxidant-deficient or exposed to increased levels of oxidative stress, for example, smokers, diabetics and elderly patients, emphasising the importance of subgroup targeting. Through defining the right

population group and the optimal vitamin combination we could potentially find a future role for vitamins in CVD (Honarbakhsh and Schachter, 2008).

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Chapter 2

Native Medicinal Plants used in the Ethnomedicine of the Córdoba Hills in Central Argentina: Relevance and Interest for Primary Health Care and Conservation

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Abstract

This study reviews the main native medicinal plants that compose the pharmacopoeia of the highland population in the province of Córdoba, central Argentina. From a methodological point of view, we combine first-hand information from previous investigations, field documents on medicinal species and their applications, and the results of other ethnobotanical studies on the region. We provide an extensive list of species and applications, a thorough description of the habits and therapeutic practices in which the species are used, and present the most characteristic features of peasant ethnomedicine. We also describe the main specific features of the etiological explanations given for diverse maladies and different forms of diagnosis and treatment. Based on the use of quantitative indicators such as the number of uses, the consensus and relative importance for a particular use and pharmaco-botanical information, we indicate

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the native species that would be interesting to apply in primary health care. Finally, we suggest practices regarding the conservation of these species taking into consideration their distribution, ecology and botanical status.

Introduction

The health policies of Argentina are currently determined on the basis of "biomedicine", also known as "scientific medicine", "occidental medicine", "academic medicine", "medical science" or "official medicine"; in other words, medical attention in hospitals, dispensaries, private practises and health care centres, where illnesses are treated based on an individual biological cause and therapies are characterised by their pragmatic efficiency (Comelles & Hernáez, 1993). However, as in many other underdeveloped countries with a multicultural tradition, a considerable part of the Argentine population resorts to "traditional" or "popular medicine", with practices based on a deeply engrained system of beliefs and with an extremely different point of view from the prevailing biomedical model or "hegemonic-medical model" (Menéndez, 1992a). In this sense, the World Health Organization (WHO) has developed a strategy that contemplates any possible contributions from the traditional medical system in reducing the mortality and morbidity, especially in low resource communities (WHO 1978a,b; 2002). This strategy involves developing policies to integrate traditional medicine with national health care systems; promoting strategic studies to ensure its safety, efficiency and quality; increasing its availability and affordability, with an emphasis on access for poor populations; and promoting its therapeutically sound use (WHO, 2002).

One of the most characteristic features of traditional medicine is the use of plants for treating health problems. Herbal medicine has been an essential part of the health system in many traditional societies. Nearly 80% of the world population resorts to traditional medicine for treating their diseases, which is mainly based on the use of extracts and active substances from medicinal plants; two-thirds of these plants come from underdeveloped countries (Alonso, 1998; WHO, 1978a,b). In the declaration of Alma-Ata, WHO insists on the need to reassess the use of plant pharmacopoeia in health care (WHO, 1978b). Apart from WHO, other international organisms like the World Wildlife Fund (WWF) also emphasize on the urgency and benefits of protecting these floral resources, as announced on different occasions like the Chiang-Mai Declaration in Thailand or at the Biological Diversity Convention in Indonesia (WHO, 1982; WHO, 1988; WHO-UICN-WF, 1993), especially regarding the emerging environmental problems.

The directives on the conservation of medicinal plants indicate, among other aspects, the need to "obtain detailed information on the medicinal species of each region and also on the indigenous communities that have known and used them in the past", as "no measures of protection have been established for most of the endangered medicinal plant species".

From an economical point of view, the medicinal flora of central Argentina is a valuable and widely used resource. Almost 40 autochthonous species are used industrially, mainly in plants processing "yerba mate" (mate tea) compounds, pharmaceutical laboratories, pharmacists, herbalists, health food shops, body care shops, cosmetology, aromatherapy and

distilleries manufacturing a variety of products like non-alcoholic beverages and appetizers (Noher de Halac *et al*., 1986; Lagrotteria *et al*. 1986, 1987a, 1987b; Lagrotteria & Toya, 1987; López, 1996; Lagrotteria & Affolter, 1999). Over the last years there has been a change in consumer preferences associated with the boom of phytotherapy and herbal medicine that has increased the number of people choosing natural products in foods, medicines and cosmetics. These and other reasons such as not including its ecological and social value, the lack of knowledge on the cultural norms involved in the use of these species, the degradation of habitats, and the absence of an adequate legal framework regulating the extraction of these species, has increased the extractive pressure on wild species with a subsequent reduction and loss of genetic diversity (López, 1996). This situation has left at least twenty endangered species (Noher de Halac *et al*., 1986).

On the other hand, the traditional cultures of peasant and indigenous communities, who hold most of this knowledge on the natural environment, are not exempt from the current world globalization context that threatens the integrity of their customs, identity and even their existence. This knowledge, developed empirically or passed down through generations, is an important element of traditional medicine. In addition to information on therapeutic properties, it includes details on the norms and criteria for collecting or propagating species in domestic herb gardens, the ecology and phenology of species, and the cultural value and symbolic significance bestowed on certain plants by the community.

Taking the above into consideration, it is clear that the protection of these resources is only possible by combining botanical, ecological and anthropological understandings. This type of interdisciplinary investigation involves specific details on plant species and the cultural norms of the people using them, both specific **ethnobotanical** topics (Barrera, 1979; Alexiades, 1996).

Arenas (1997) defines **ethnobotany** as the study of the reciprocal relationship between man and vegetation; in this sense, it is understood that instead of a discipline it is an "interdisciplinary field" that interprets the knowledge, cultural significance, management and traditional uses of the elements in a flora (Caballero, 1979). The specific interest in medicinal flora is a topic of ethnobotanical medical studies, which use these articulate resources within the framework of the representations and practises of health and disease of a particular human community, in other words, within the context of their ethnomedicine[1].

Although the bibliography on the use of plants in the traditional medicine of Argentina is vast, it mostly relies on repeatedly quoted sources based on unknown survey sites and human communities (Hieronymus, 1882; Sorarú & Bandoni, 1978; Ratera & Ratera, 1980; Toursarkissian 1980; Marzoca, 1997; Lahitte et. al. 1998, Barboza *et al*., 2001, 2006; among others). Specific studies on medicinal plants in central Argentina are less common, but they have gained increasing interest over the last decades. The information available on Córdoba is referred to the Department of Río Cuarto in the south of the Province of Córdoba (Bocco *et al*., 1997; Núñez & Cantero, 2000) and other technical reports (Noher de Halac *et al.*, 1986; Lagrotteria *et al.*, 1986, 1987a, 1987b; Lagrotteria & Toya, 1987; López, 1996; Lagrotteria & Affolter, 1999). Martínez (2002) and Arias Toledo *et al*. (2007) analyze the intergenerational

[1] We follow the criteria of Comelles & Martínez Hernáez (1993) who consider ethnomedicine to be based on anthropological investigations while medical folklore is referred to compilations on traditional medicine carried out by physicians.

knowledge on medicinal plants in the localities of Valle de Paravachasca in southeast Córdoba, as well as issues related to their harvest and commercialization; however, these studies have a more quantitative focus rather than a strictly ethnomedical view (Martínez, 2005a). The report by Barboza *et al*. (2006) constitutes a highly valuable compilation effort and no doubt is the most complete compendium on the medicinal plants of the province. All these aforementioned studies detail floristic aspects, description of species, lists of medicinal plants or commercialization problems. Nevertheless, none of them has focused their attention on the ethnobotanical and ethnomedical aspects that allow to interpret the context in which many of these plants are used; on the contrary, most of them mention the medicinal uses and applications of different cultural contexts and communities in the country (indigenous, peasant and urban populations), without specifying the *in situ* uses given to these plants by the local inhabitants.

Objectives

The general objective of this article is to review the main medicinal plants that comprise the pharmacopoeia of the inhabitants of the central Argentine highlands, particularly the Sierras de Córdoba (Córdoba hills), with special emphasis on their importance in medical practises, interest for conservation and possible applications in primary health care.

Thus, we will first describe the outstanding features of peasant ethnomedicine that enable a better understanding on the therapeutic practices and modalities in which they are used. We then present a list of species and their applications, floristic composition and ethnobotany, underlying the relevant native species that would be interesting to apply in primary health care. This is based on first hand information obtained by the authors in investigations and field studies documenting the species, and on other ethnobotanical investigations developed for the region. Finally, we describe the current state of investigations regarding the conservation and propagation of the medicinal flora of the *serrano* (highland) environments. At the same time, and considering the distribution, ecology, botanical status and/or extraction/commercialization pressure of these species, we point out considerations regarding their conservation.

Traditional Medicine in Peasant Populations of Argentina

The ethnomedical investigations on peasants in our country have been particularly interested on the *criollo* and *mestizo* populations in northwest Argentina (Márquez Miranda, 1949; Palma, 1973, 1978; Perez de Nucci, 1988; Hurrel, 1991; Bianchetti, 1989, 1996; Idoyaga Molina, 2000a,b; 2001a,b; 2002; 2003). There are also references to the littoral communities of northeast Argentina (García, 1984; García & Jiménez, 1986; Jiménez de Puparelli, 1984), centre and west of the Province of Formosa (Sturzenegger, 1989; Scarpa, 2004a,b), and to peasants in the Cuyo region in central-west Argentina (Idoyaga Molina 1999a,b, c; 2001a,b; 2003; Idoyaga Molina & Krause, 1999; Krause, 1999). Likewise, there are many reports with extensive descriptions on the lexicon of diseases, and an interesting

repertoire of remedies, practices and cures of the medical folklore of central and north Argentina (Di Lullo, 1944; Sosa Verón & Vivante, 1950-1951; Carrizo, 1960 and others).

Emphasising the ethnobotanical point of view and methodology, Martínez Crovetto (1981), Arenas & Galafassi (1994), Scarpa (2000a,b, 2002, 2004a,b), Hilgert (2001) and Nicola (2002; 2006) have carried out field studies within an ethnomedical framework, with *in situ* documents on the species and uses of the plant pharmacopoeia of peasants communities in Corrientes, Chaco, Salta, north of Santa Fe and other regions of the country. They support their information by collecting herbarium specimens and limit their investigations to a culturally defined group.

Compared to other areas of the country, the knowledge of the rural inhabitants of central Argentina, particularly the province of Córdoba, has hardly been studied from an ethnobotanical and ethnomedical point of view. Basically, we can mention the work of Arias Toledo (2006) and Menseguez *et al*. (2007) in the Department of Tulumba, the work of Goleniowski *et al*. (2006) in the Comechingones hills and the studies of Martínez (2003, 2005b, 2007, 2008a,b) and Martínez & Planchuelo (2003) in the valleys of Paravachasca and Calamuchita in the south of Córdoba.

Within the context of a multiethnical and pluricultural country like Argentina, the traditional medicine of peasant communities is part of a complex ethnomedical system that coexists and sometimes even replaces "official medicine" or biomedicine, as well as other alternative and religious therapies (Idoyaga Molina, 1999b,c, 2000b, 2003). Likewise, and as mentioned by this author, it is possible to identify three types of traditional medicine practices: shamanism, healers and home remedies or self-treatment. The first is still in force in indigenous communities, while the other two are practiced in the non-indigenous *criollo* population both in rural and urban areas.

Healing practices and home remedies have well-defined specific features regarding the notions of disease, the way of naming and classifying maladies, methods of diagnosis and therapeutic practices. This is because they are based on the same traditional cultures originated by different combinations between indigenous knowledge and European beliefs, beginning at the time of the Spanish conquest (XVIth century), and reinforced later on by the European immigrants of the XIXth and XXth century (Idoyaga Molina, 2000b, 2003).

One of the most documented aspects among the non-indigenous populations of America that is observed in these practices is the presence of principles reformulated from humoral medicine, and the notions of "hot" and "cold" for classifying diseases, foods and therapies, and even plants (Foster, 1994; Idoyaga Molina, 1999a, 2000b, 2003). Based on these theories, it is believed that an imbalance caused by excessive heat or cold is involved in the etiology of many illnesses; thus, the subsequent therapies try to re-establish this balance by using "hot" or "cold" plants. This knowledge, together with other practices of Spanish popular medicine like the "*cura por el rastro*" (healing by a person's tracks) and healings by words and prayers, constitute the cultural base of the present **healing practice**s. This type of practice also involves the knowledge, diagnosis and treatment of traditional ailments like "*empacho*" (indigestion), "*ojeadura*" (evil eye), "*susto*" (fright), "*envidia*" (envy), "*daño*" (harm), "*pata de cabra*" (goat foot), "*culebrilla*" (shingles) and "*mal aire*" (bad air), the significance of which will be explained in the chapter on etiology.

While consulting specialists or "healers" is not unusual, many inhabitants also know and use different home remedies based on plants, animals and minerals through family tradition, which constitutes another form of traditional peasant medicine: **self-treatment, domestic medicine** or **home remedies** (Idoyaga Molina, 1999a, 2000b, 2003; Zolla *et al*., 1992; Menéndez, 1992a,b).

As to our knowledge there is no native classification for ailments, we will remit to the suggestion of Idoyaga Molina (2003) who, based on a causal perspective, interprets disease as a consequence of five possible types of imbalances. From this perspective, traditional medicine focuses on the patient before the disease, which is why the latter is fundamentally interpreted in terms of disharmony, imbalance or disorder by an excess or deficiency of some sort of agent or factor. From the viewpoint of this author, the diversity of nosological entities can be interpreted as a manifestation or consequence of some type of imbalance listed below. The first two describe most of features of peasant medicine, while the remaining are referred to etiologies from other countries.

- Organic imbalances: manifested only in the body and originated by natural causes such as nutritional imbalances[2], temperature imbalances, strains and bangs, and blood or skin alterations.
- Social imbalances: caused by conflicts in social relations like "*envidia*" (envy) and "*daño*" (harm).
- Imbalances between the entities that form an individual, in other words ailments produced by a disharmony between body and spirit.
- Environmental imbalances: disorders produced by negative environments.
- Religious-ritualistic imbalances: originated by the transgression of taboos or disturbances in the relations with mythical beings, expressed in physical and psychical manifestations as well as other ailments.

Ecology, Plant Resources and Highland Culture

From an ecological point of view, the information we present in this report belongs to the ***Chaqueño Serrano District*** (Chaco Province) that extends, from North to South, from the main mountain ranges of east Jujuy, down through the centre of Salta and Tucumán, east of Catarmaca, and continues south to the hills of La Rioja, San Luis and Córdoba reaching a latitude of 33º S (Cabrera, 1994). The variety of highland environments in central Argentina, particularly in Córdoba, produced by the orogenic movements and geomorphological processes that sculptured its relief have promoted the coexistence of species of the most diverse floristic types. These include Andean elements (i.e. *Polylepis australis, Fagara coco, Lithraea ternifolia, Kageneckia lanceolata, Flourensia, Plantago, Phacelia*), Andean-Sonoran elements (i.e. *Caesalpinia gilliesii, baccharis, Lycium, Ephedra*) tropical elements (i.e. *Goeffroea decorticans, Celtis spinosa, Porlieria microphylla, Nicotiana glauca, Jodina*

[2] Imbalances originated in nutritional imbalances are expressed by different symptoms like fever, stomachaches, headaches, indigestion, etc., and are generally caused by eating excessively. Temperature imbalances cause respiratory problems, flu, colds and other digestive disorders.

rhombifolia), Austro-Antarctic and endemic elements (Luti *et al*., 1979). Occupying the lower slopes of hills and ravines, and distributed along an altitudinal gradient that encourages the presence of different layers with typical species ("*Bosque Serrano*", "*romerillal*" or highland grasslands or forests), the Chaco Serrano area is characterised by a dominant xerophytic forest interrupted by grasslands at higher altitudes.

Similarly, the cultural and socioeconomic dynamics of the *serrano* inhabitants links them to other nearby regions that also provide medicinal resources; such is the case of the neighbouring piedmonts or valleys which, in addition to the variety of highland species, comprise an area of great diversity as here the Chaqueño Province merges with the Espinal and Pampeana Provinces.

From a cultural point of view, especially regarding the way of envisaging health, disease and cures, this study is referred to the knowledge and practices shared by most of the *criollo* [3] settlements that compose the peasantry of central Argentina. The work of Idoyaga Molina (2003), based on many descriptions collected from towns of central and northern Argentina, from Jujuy to San Juan, shows that there is a homogenous concept on the notion of illness in the way peasants represent organic imbalances (temperature imbalances, digestive, skin and blood disorders like "*cullebrilla*", "*empacho*", "*nervios*", "*pata de cabra*" and social imbalances ("*envidia*", "*daño*", "*brujería*"). On the other hand, there are conceptual variations on illnesses regarded as ritual imbalances depending on whether the indigenous or European beliefs prevail; thus, according to the aforementioned author there are three different sub-areas:

- A northern sub-area (Puna region, valleys and ravines of Salta and Jujuy), with a strong influence of indigenous traditions where the ritualistic notion of the Pachamama (Mother Earth) and maladies like "*sopladura*", "*agarradura*", "*pilladura*" and "*aikadura*" are important.
- A central sub-area comprised by southern Salta, Tuchman, Catamarca and Santiago del Estero, where indigenous representations gradually loose importance and European beliefs gain entry, and where practices related to the Pachamama are less common.
- A southern sub-area (La Rioja, San Juan, Córdoba), referred to in this review, in which the indigenous influence is practically non-existent and where traditional Catholic and other European representations become dominant, associating ritualistic imbalances with popular saints.

Even though the main focus of this chapter is the ambit of traditional medicine that characterizes the peasant culture of *serrano* environments, it is necessary to point out that it is embraced within the framework of a wider ethnomedical system, where healing practices and

[3] In accordance with Idoyaga Molina (2001a), the term "*criollo*" is used in this study "to designate peasants of European descent, in the same sense as it was used in the XIXth century to difference Europeans born in America (the *criollos*) from Europeans born in the Iberian Peninsula and other Old World countries". Hence, the meaning of *criollo* proposed here is different from the meaning of Creole in other American countries that is always associated to mixed races or "*mestizos*".

other home remedies are articulated with the attention provided in primary health care centres and other official medicine ambits, as well as with places dispensing alternative medicines.

For more specific information on the environments and inhabitants of each of the regions considered in this review, we suggest the reader to consult each of the individual studies cited in this analysis shown in Figure 1.

Methodological Aspects

[1] The present review is based on investigations supported by pluri-methodological approaches combining quantitative and qualitative techniques. It is divided into three thematic sections:

I. From the perspective of a qualitative investigation with an ethnographic approach, we present the medical ethnobotanics of highland *criollo* peasants based on a series of studies performed between 2001 and 2007 in the intermountain valleys of Paravachasca and Calamuchita in the Sierras Chicas region of the province of Córdoba (Martínez & Planchuelo, 2003; Martínez, 2005b, 2007, 2008a,b). In this section we interpret the use of medicinal plants and other remedies, not only describing the uses given to plants but also trying to understand these practises considering the particular way the peasants of this region conceive, diagnose and treat diseases. Within this context, and in addition to the documented collection of plant specimens, the therapeutic practices and plants used by more than 60 local inhabitants were registered by open and extensive interviews, semi-structured surveys and data from participative observations. Although this work is based on a regional study, we obtained similar information in experiences and encounters with inhabitants from other regions of central Argentina, showing that this study documents a characteristic and genuine expression of traditional peasant medicine of highland environments. Consequently, the content of this review is extensive to all the inhabitants of valleys and hills in our province.

II. A summary of medicinal plants and their applications obtained from ethnobotanical investigations carried out in the province of Córdoba. For this we developed a database to catalogue the medicinal species with their corresponding applications and regional uses. The studies included in this analysis were strictly ethnobotanical, in other words they were based on information obtained by interviewing local inhabitants before documenting the herbarium specimens sustaining the results. These specimens were grouped according to the geographical location of the surveys, which belong to the following four regions of the province of Córdoba (**Figure 1**):

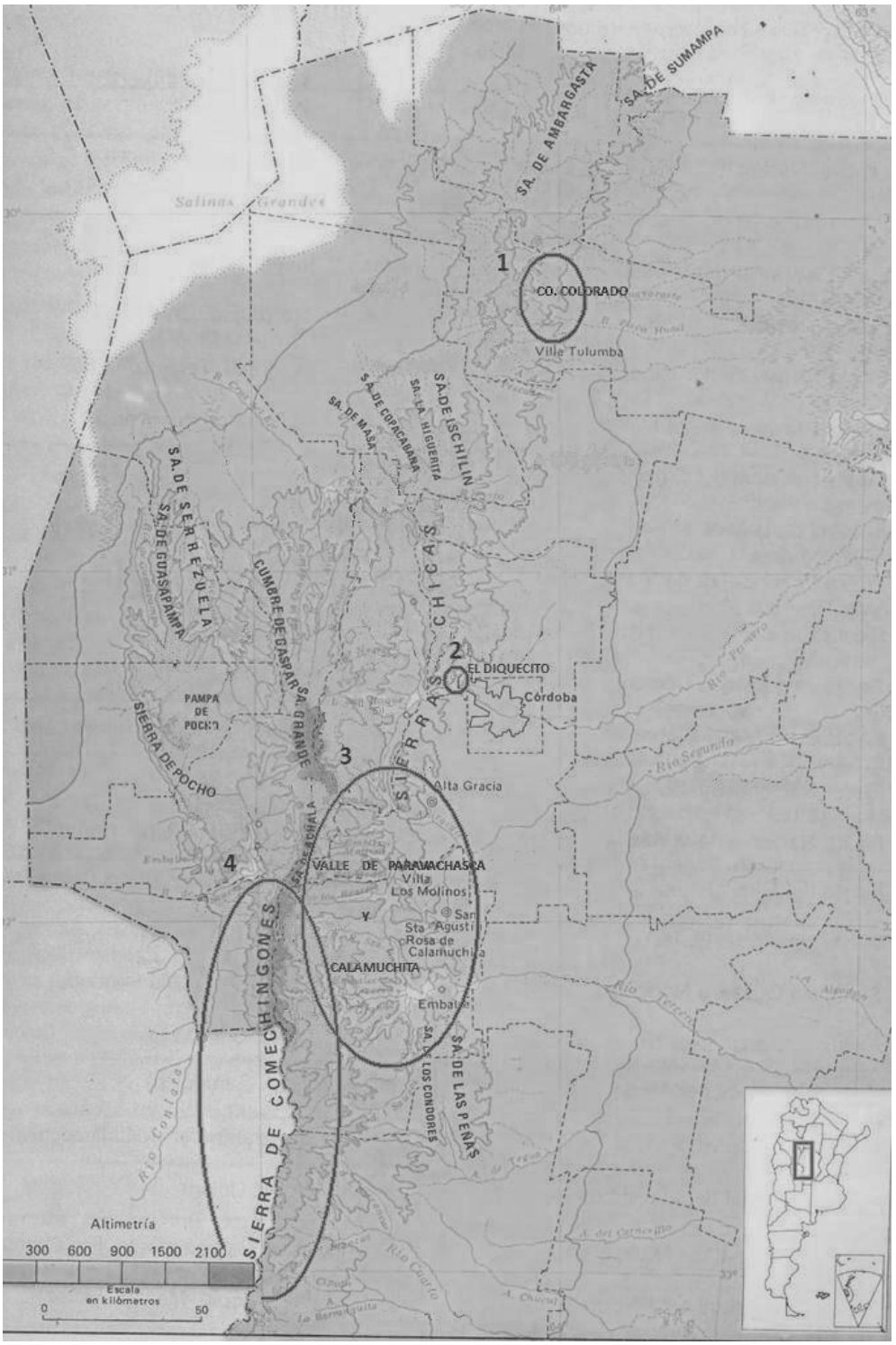

Figure 1. Geographical location of the study area, belonging to the following four regions of the province of Córdoba: 1) Northern Region: Cerro Colorado, Departament of Tulumba. 2) Southwest Córdoba: Sierras de Comechingones; 3) Region of Paravachasca and Calamuchita in the Sierras Chicas of Córdoba; 4) Western Region: "El Diquecito" Department of Colón.

(1) Northern Region: the information gathered by Menseguez *et al*. (2007) and Arias Toledo (2006) is referred to the Department of Tulumba in the Cerro Colorado area, which has a characteristic Chaco Serrano vegetation.
(2) Sierras de Comechingones Region, Southwest Córdoba: based on an ethnobotanic study performed in the Comechingones hills (Chaqueño District) in the limit with the Province of San Luis (Goleniowski *et al*., 2006).
(3) Region of the southern hills and valleys: based on investigations carried out in the valleys of Paravachasca and Calamuchita in the Sierras Chicas of Córdoba (Martínez, 2003, 2005b; 2007; 2008a,b; Martínez & Planchuelo, 2003; Arias Toledo *et al*., 2007).
(4) Western Region: based on information from the locality of "El Diquecito", to the west of the city of Córdoba, in the Department of Colón. This is a semi-rural hilly area that is progressing towards urban characteristics. Although the ethnobotanical information is scarce, fragmented and still has to be systematized, we considered preliminary data obtained in a participative project in a rural school (Salguero & Asad, 2006).

A comparison of the medical uses and applications was performed between the different regions following the criteria of intercultural comparison proposed by Heinrich *et al*. (1998). The following estimations were made using quantitative indicators:

- Recurrence of a specific medicinal use or application of a plant in the different regions of Córdoba considered in the study.
- Inter-regional agreement on different categories of medicinal use for the most cited species, evaluated by the Informant Consensus Factor (F_{ic}) according to the following formula:

$$F_{ic}= (n_{ur}-n_t)/(n_{ur}-1)$$

where n_{ur} is the number of use-reports in each category; n_t is the number of taxa used; and n_{ur} is the number of use-reports in each category. This factor ranges from 0 to 1, where a high value (near 1) indicates that relatively few species are used by a large proportion of informants and a low value indicates that the informants disagree on the taxa used in the treatments of a certain illness category (Heinrich *et al*., 1998).

III. Taking into account the distribution, ecology, habitat and origin of the implicated medicinal species, as well as information on their harvest, extraction, use and commercialization, we suggest methodological and technical considerations as well as practices destined to the *in situ* and *ex situ* conservation of medicinal flora.

[2]

Results and Discussion

I. Health and Medicine of the Highland Peasants

In this section we underline the role of healers and home remedies in the highland communities. Thus, we describe the local names of illnesses, their representations, etiology and their corresponding diagnostic and therapeutic practices. Likewise we attempt to comprehend, from an anthropological point of view, the logic of the practises used to prevent and cure ailments, making special reference to the role of plants and other remedies in this context.

1. How Peasants Name, Diagnose and Explain the Causes of Different Ailments

A- Organic imbalances (originated by natural causes)

Originated by temperature imbalances

- *"Pasmo"* (Spasm)*:* this is one of the most commonly mentioned affections among the local inhabitants. It is described as a body spasm due to sudden changes in temperature between the body's heat and cold air, or by being close to sources of heat. When the chill or "*pasmo"* is produced in the head it causes headaches, whereas if it is in the chest is causes respiratory problems like coughs, colds, bronchitis or pneumonia. Within the context of Hippocratic notions, it is caused by both hot and cold agents. In the former case, the affection is similar to the description of Arenas & Galafassi (1994) for "air". However, some informers believe that hot agents are the cause of "*pasmo"* in the legs, manifested by tiredness and pain, or the one producing toothaches, as they are generally associated with inflammations caused by being near heat sources. Some people difference the types of "*pasmo"* according to the direction of the wind (North or South) causing it.

"You leave a warm place and go outside and get a cold that you don't feel at the time, but after a while it has repercussions all over the place, in your bronchi…"

"…its a sort of inflammation, its swollen, when you go barefoot you get "pasmo" and I suffer from the kidneys, I get an inflammation afterwards"

"I know what a "pasmo" in the chest is: for that I give honey with salt and "pulmonaria" which I boil a bit…and afterwards I put that water where the honey and salt are, and the towel heats up and then its placed on the chest for that to spread"

"Pasmo is something that just gets you –how can I explain: You get wet, feel cold, like when you are about to get flu, as if you were about to get a temperature, afterwards you feel weak, sleepy, you want to sleep and your eyesight goes cloudy, it stings a bit…For example, there are 2 or 3 types (of "pasmo"), not only one. Like

stings in bangs, "pasmo" is that which goes all red, that line…like a red line, I don't know whether you've ever noticed it in a bang or in a sting, well: that is "pasmo". Its very dangerous… you have to stop it as soon as possible because it can cause a heart attack"

The explanations given by the interviewed subjects regarding these disorders mention the imbalances caused by thermal agents associated to the "hot-cold" classification system. In general, the affections produced by drafts when the body is hot or by differences in temperature between the body and the environment are considered "cold" illnesses (colds, flues, cough, catarrh, earache, diarrhoea, and in some cases "*pasmos"*). Ailments in bones and muscles as well as paralysis are also attributed to the effects of cold temperature..

"Pain in bones is caused by cold and the "peje" is hot…is has two thorns, it is a plant of the hills"

"Fresh is anything that contains cold and does not alter your pressure or anything else in the body"

On the contrary, ailments produced by exposure to external (sun, embers, stoves, ovens) or internal (fever, eating indigestible food) heat sources are "hot" (odontalgia, haemorrhoids, varicose veins, pains in legs, sun-stroke, some cases of "*pasmo"* and "*empachos"*).

Likewise, loss of blood makes people susceptible to getting fresh diseases; thus, a woman is more vulnerable to gyneco-obstetric cold diseases if she is menstruating or a in a postpartum stage (Anderson, 2004; Randall, 1993). The insistence for recent mothers to avoid contact with water is explained in the same way and if this norm is not abided it impedes the ability to breastfeed and causes severe illnesses that can even put the mother's life at risk. This is evidenced by the following comments of a midwife:

"If you get wet during the 40 days after delivering a baby your next birth comes out wrong. Only after 40 days. Nowadays babies are put into water as soon as they are born. That is where the evilness of people comes from; there is so much illness now, why? Because the women have baths…that is where all the diseases come from, cancer and all that that comes out in women's breasts. Because they get wet, but the people don't understand this; as soon as they give birth they are having baths. My mother would advise me never to get wet when I had the baby. You couldn't wet anything, your body, your face, anything for 40 days. If not it is bad for you, you get haemorrhages."

"For example if you wet your hands when the baby is born your milk dries out, the person's milk disappears, it is "cut". That is why people take care, so as not to get lockjaw…if you don't take care for 10 or 12 days. The lockjaw is a chill you get, and that chill goes to your back. It has happened to me. If you wet your hands or wash your face, that is where you get the draught of air. Before they would rub your back with a slab of camphor oil to calm the spasm so the milk wouldn't dry out. Now nobody does that."

- *Falso cruz or Falzo crup* (False croup): this is characterised as a beginning of asphyxia that generally happens in children due to an inflammation in the thoracic region, a "*drop in*

pressure" or by exposure to cold, a situation diagnosed by measuring the back with a tape. Associated or not to this affection, other respiratory diseases are also originated by a "*golpe de frío*" (chill) that many times causes a cough, "*pain or stitches in your side*", a symptom indicating *"badly cured bronchitis", "pneumonia"* or *"pleurisy"*.

Originated by food imbalances

In this area, as in other parts of the country, the following diseases are repeatedly mentioned, especially regarding ailments in children:

- "*Empacho"*: it is interpreted as an ailment affecting the digestive process, originated by excess food or by eating some sort of indigestible preparation.

"It is when you eat a food that does not suit your stomach, a heavy food… fried food, sauces, "puchero" (meat stew)"

"when they are ill and lying there, vomiting… I was intoxicated with cold meats… she cured me, she pulled my skin and the "empacho" becomes unstuck, that badly cured "empacho" which afterwards caused meningitis…he was taken out of hospital

"it is when you eat and the food remains in the stomach, it can be due to cold, if the stomach was cold, and if it is fat it is worse"

This ailment can be produced together with a series of symptoms like headaches, diarrhoea, vomits, loss of appetite, constipation and fever, and can also be originated by a temperature imbalance (caused by cold or a "*pasmo"*). In accordance with the report of Jiménez de Pupareli (1984) on the peasants of the Paraná, we found that the inhabitants of the area give these types of affections special and frequent attention.

- "*Fiebre intestinal*" (Intestinal fever): it is a particular type of "*empacho"* characterized by the presence of diarrhoea, which is produced by eating warm or green fruit or by sitting on hot places (rocks, seats, etc.).

- "*Pata de cabra"* (Goat foot): an ailment that affects children and, as described in the interviews, is revealed by the appearance of one to three marks like bruises with the shape of a goat's hoof on the back of the body (usually on the hips, back or cervical region). Among other symptoms, it causes children to arch their backs, turn their eyesight backwards and become cross-eyed, and produces vomits and diarrhoea with greenish faeces. According to the informants, the child struck by "*pata de cabra"* looks gaunt, cries, trembles and kicks aggressively. Furthermore, if unattended it can end in serious conditions like meningitis and can even be fatal. The peasants describe many causes for this disease, most of them related to feeding, changes in the children's milk, badly cured "*empachos"* or the effect of pathogenic agents.

"When it is not cured, the "pata de cabra" causes meningitis, because the stomach's overload is not cleaned out unless you give them (the child) a tea or some sort of purgative…" "…you realise because they have a bad stomach, they become

cross-eyed, they get rheum, the "empacho" is very high up and it harms them" ... "it is not known what it is, a bug or a worm" "...when the "pata de cabra" becomes too much they die flaccid, dislocated. When you get the "empacho" measured and it gets a bit better and it is up to the head or goes towards the back, it becomes a high "empacho"
"They say that it is from a badly cured "empacho" or because they change the milk of babies with constipation..." "they (the babies) begin to dehydrate, their eyes become sunken and they arch back to cry"

"It is a microbe that lodges in the baby's spine and slowly eats the medulla. They arch back and cry, cry and cry. It is only cured by a healer, older people that understand about that, not by doctors."

"It is something that children get in their bottom, it looks as though it were a bruise, it has the shape of a foot, a little bruise...it is terrible for them, it moves up the spine..."

Originated by blows or strains

The most well known reference in the area is "*nervios"* (nerves). This ailment involves the extremities and its most characteristic symptoms are pains, limping and difficulty to walk.

"*They are veins that get out of place..." "the tendons get out of place", they are produced by "making a bad effort or by stepping falsely" and "they hurt like a sprain".*

For some informers a *"golpe de aire*" (chill) can also cause nerves, in which case it is interpreted as a cold disease originated by temperature imbalances.

Another expression among the *criollos* is the allusion of *"pain in the bones"* which is generally referred to the extremities. Occasionally some inhabitants mention "*paletilla"* as a "*type of fever that you get which remains in a art of the body without movement"*.

Originated by alterations in the properties of blood and body humours

For the *criollos*, the condition and quality of blood are significant indicators of a body's general health condition. Furthermore, alterations in blood properties produce a diversity of symptoms affecting other systems, mainly the skin (Queiroz, 1984; Scarpa, 2004a). This is evident in the expressions used by the inhabitants of the hills, who believe that "fat", "thick" or "dirty" blood is a consequence of eating fat rich food or having done some kind of excess that disrupts the composition of blood. As mentioned in other studies (Scarpa, 2004a), the balance from these disorders is regained by taking blood-depurative plants that are able to "thin" "purify" or "clean" the blood. The depurative effect of the plants is not only evidenced by an improvement in the blood conditions, but also in the healing of skin affections such as pimples or "*dibiesos"* (furuncles) that are believed to be caused by blood alterations. In this sense, the informers mention that blood impurities are treated using these depurative beverages and that balance is regained once the impurities are eliminated. The benefits of drinking these depurative teas, especially during August, have also been mentioned. It is thought that during this month the body undergoes a natural depuration process due to

changes in the blood. These kinds of expressions and physiological explanations in popular terms evidence a Hippocratic-humoral conception of blood, a feature shared with the traditional medicinal practices of peasants from other regions of Argentina (Scarpa, 2004a; Idoyaga Molina, 2001a) and of the Americas (Queiroz, 1984).

Originated by agents affecting the skin

According to the informers, "*culebrilla"* or "*culebría"* (shingles) is a disease produced by an iguana or small lizard that is generally invisible when it acts. The afflicted person suffers a sort of burn that goes round their waist. If this eruption closes round the waist it is very dangerous and can lead to death.

"It is by the urine of a "culebrilla", while that animal walks the rash grows and if the ends of the rash meet in the stomach it is very dangerous because the infection is passed on to the intestines… it is like a very small iguana, there are two types, one is black with white spots and the other is green with two yellow stripes down the side to its tail… it contaminates the clothes line, it urines on the clothes that you left in different places… It is cured with prayers and Indian ink. The wound is surrounded for it not to walk".

"It is a small bug, no one has seen it, it is an allergy that causes rashes, small ones that advance, that produces fever, stinging and itching. "Culebrilla" is cured with Indian ink, the black one… you have to cut its progress, surround it with ink"

Curing the irritating and harmful effects of snake, arachnid (spiders and scorpions) and insect bites is a common matter for the older practitioners; in this case, the efficacy of the remedies, plants or preparations is mainly symbolical rather than physiological or pharmacological. They also mention injuries, irritations and burns produced by the sun or by natural caustic or allergenic substances as for example the "*flechaduras*" or "*canchas*" (rashes or skin ulcers) caused by exposure to the "molle" tree (*Lithraea molleoides*). Other ailments produced by this cause are interpreted as disorders originated by temperature imbalances, as is the case of sunstroke.

Although less known, the "*mal de pie*" (foot malaise) is an ailment probably associated with mycosis, as explained by one of the specialists: *"The whole foot is covered in blisters underneath, as if it were a fungus. That is not cured by a doctor"*

The additional details and testimonies that refer specifically to skin conditions, as well as a detailed list of plant pharmacopoeia recipes for their treatment, can be found in the work of Martínez (2008b).

Other imbalances and ailments

Other etiological explanations use elements of biomedical language. For example, "*culebrilla"* is produced by the patient's contact with the urine of a lizard or "bug" that is not seen, but that is the carrier of the "virus" or "microbe" causing it. Using different terms, the

interviewed individuals mention ailments of different parts of the organism, making specific reference to a precise part of the body. Among the commonest are "*nubes*" (clouds) or "cataracts" interpreted as a "*little web that appears in the eye"*, "*mal de orín"* (urine disorder) and "*arenillas*" (sands) in the kidneys, "*chuchos*" (shivers), "*chuschamientos*" and "*calores"* (heat flushes) or "*fevers*", all of which are considered locatable morbid agents rather than symptoms of an imbalance.

In addition to the characterization of symptoms for the aforementioned afflictions, a *diagnosis by urine or faeces* is customary. According to Di Lullo (1944) and Foster (1953), this practice is inherited from Spanish medicine, and is one of the most common forms of diagnosis used by the *serranos.* The colour of urine allows identifying kidney and digestive disorders, and making a prognosis for the patient, among other things. The precision of this "diagnosis by the waters" or "reading of the waters" is evidenced in the narration of an elderly informer capable of identifying different types of "*pasmo"* according to the direction of the wind.

> *"My father saw the waters, in other words the urine doctors take to study… they bring him a clean bottle; my father would put it into a crystal bottle and look at it over a white sheet of paper"*
>
> *"Brown and dark coloured urine, like Coke indicates a kidney problem" "urine the colour of Coke is hepatitis, when the urine is lightly coloured it is healthy"*
>
> *"It is known that the milk has done them harm, I look at them and then look at their urine, if the urine is very strong, almost like Coca-Cola"*
>
> *"When they have "pata de cabra" they defecate green"*
>
> *"A rubbery and light green faeces is due to a cold or intoxication with phlegm; that is cured with the tape measure, "palo amarillo" tea or another tea with a laxative effect… greenish defecations like a beaten egg is a liver problem…urine with dark blood is kidney problem that they are not working properly"*

B. Imbalances generated by conflictive social relationships

Many symptoms of the *criollo* diseases are often interpreted as direct consequences of conflicts related to social relationships, expressed in terms of negative energies, forces or supernatural powers.

Evil, envy, harm and sorcery are often mentioned in the etiology of diseases of newborn babies and heart or nervous affections. This is especially evident when explaining the causes of asthenia, loss of weight and appetite, insanity or other symptoms as irritability, nervous anxiety, insomnia and even sudden death due to heart failure. In this area these signs are diagnosed as an ailment known as "*ojeadura*" or "*mal de ojo*", meaning "evil eye". According to the peasants' beliefs, this ailment reflects the fact that some people can cause harm by the way they look at others, whether intentionally (by praise, envy, or greed) or not (a relative or neighbour that looks fixedly at a weak person after a long-working or troublesome day). "Evil eye" often affects children, especially newborn babies that are weaker than adults and get ill when exposed to a more powerful energy. "Evil eye" is also

found in other regions of Argentina (Di Lullo, 1944; García & Jiménez, 1986; Arenas & Galaffassi, 1994; Idoyaga Molina, 2001a,b, 2003; Disderi, 2001) and is deeply rooted in Hispanic-European traditions (Kuschik, 1995; Pieroni, 2002). The diagnosis and therapy for this ailment is reserved to specialists and generally does not involve plants. It is cured using a practice very similar to that of other areas of America and Europe: a plate with water into which some drops of oil are poured down a spoon. These therapies combine prayers and magical incantations together with gestures symbolizing the expulsion of the cause of evil, while pointing at the region of the temples. The treatment of these afflictions can also involve the use of plant species that have been attributed magical or powerful properties, as for example the use of rue (*Ruta chalepensis)* for the treatment of amnesia (Arenas & Galaffassi, 1994), or the use of "guayacán" (*Porlieria microphylla*) for the treatment of "evil eye" (Martínez, 2007; 2008a).

As evidenced in many anthropological studies that specifically refer to this topic, the action of a "*daño"* or sorcery is generally produced straight onto people, either the body itself or any equivalent (footprints, clothes, humours), or can be carried out like a contamination of the environment in which they live (houses, land, animals). The action of sorcery and its connection with illness among the peasants of Córdoba is no different, as indicated extensively in other studies (Martínez, 2003; Martínez & Planchuelo, 2003).

In summary, these types of imbalances express the tensions, and social and personal conflicts that arise from the interpersonal emotions in a community, and are translated as the effect of evil omens in the social sphere of the persons involved. An etiology of this type is frequently interpreted with afflictions that are prolonged in time and whose cause cannot be explained by the official medicine ambit, which usually gives a favourable diagnosis (Barrios, 2000). Therefore, the intervention of a specialist or healer is requested for both a diagnosis and for treatment as these are imbalances that, as the locals say, "*doctors don't know how to cure".*

2. How Peasants Prevent and Cure Different Afflictions

Although in this section I refer to the aspects related to traditional preventative and therapeutic practices, and our studies were focused on the content of the narrations rather than on the practices themselves, I must point out that in order to avoid an essentialist or static view of their medicine, the representations and praxis described here are articulated within the framework of therapeutic itineraries or surveys including the entire local ethnomedical system. In other words, apart from visiting a healer to cure their maladies, many peasants also go to dispensaries and hospitals, take aspirins, accept pills, vaccines and injections, use contraceptives, supplement treatments, practise rituals and prayers, buy plants in herbariums, obtain information of new medicines by the media, and even generate interpretations, answers and new treatments to new diseases like cancer. Hence, the traditional medicine practices I describe here are dynamic and permeable to the political influences of global health politics. Therefore, this medicine is related to other settings like official medicine, religious cures and, to a lesser degree, alternative medicine, all of which constitute the local ethnomedical system.

"I prefer to use wild herbs. I sometimes use those (pharmaceutical medicines), for example if I have a deep wound, a sting… then we put something we know is a prepared product, a very different chemical"

"Yes, I also send him to the doctor. I check him; for example I see his lungs, but all the same, if I see that the child has some sort of defect, then I send him to a psychologist or doctor…depending on what he has."

A. The use of remedies

The ethnobotanical studies we included in this review describe the use of more than three hundred medicinal species for the treatment of a wide range of ailments that characterise peasant ethnomedicine. Moreover, Barboza *et al*. (2006) estimate that the medicinal flora available in the province, whether used or not by the local inhabitants, includes over six hundred species evidencing the great value of these resources. In Section II we provide a detailed description of some of these species and present the applications with the greatest consensus between the different regions of the province.

Despite the fact that organotherapy and opotherapy (the use of organs or animal secretions with therapeutic ends) had acquired importance among the indigenous people of the region, there is hardly no evidence of the use of remedies of animal origin among the inhabitants of the Córdoba hills; however, it is popular in the northern and northeast region of the province, an area that still has scarce records on its pharmacopoeia. Additive and fragmentary references regarding the use of animal remedies describe the repetitive use of animal fats for the "*unto sin sal*" (ointment without salt) –fat from cow or pig abdomen- or iguana fat (*Tupinambus* spp.) for treating skin affections (Martínez, 2008b); the use of "*bichos bolitas*" or "*chanchitos*" (pill-bugs - Order *Isopoda;* Crustaceae) and/or worms (Order *Oligochaeta*; Annelida) for ear affections; sour lamb bile as a pediculicide; cobwebs to stop nose bleeds; boiled water with the "ant-hole flower" to treat bone pain; and egg whites for burns.

References on remedies using minerals are also scarce and mainly limited to the use of salt; also mentioned is the use of some organic inert secretions and substances like kerosene, ashes from burnt clothes, brick powder, creolin, shoe polish, among others, the details of which can be found in the work of Martínez (2003) and Martínez & Planchuelo (2003),

As in many other Latin-American peasant communities, all these therapies using natural remedies are based on the Hippocratic principal of binary opposition. As the etiology of different diseases involves an imbalance by excessive cold or heat, the therapeutic conceptions try to re-establish this imbalance by using "cold" and "hot" plants depending on the case. We will not extend in the details and lists of afflictions, remedies and therapeutic practices considered "fresh", "warm" and/or "cordial" according to the representations by the peasants of the Córdoba hills, which are already extensively discussed in each particular study (Martínez, 2003, 2005b; Martínez & Planchuelo, 2003).

B. Symbolic and ritualistic aspects of peasant cures

Although an important number of species and uses listed in the catalogue of medicinal plants of the Córdoba hills are supported by phytochemical and pharmacological studies, it is only fair to note that for the peasants, the healing capacity of a remedy is not only based on

the power of its active substances but also, and above all, on the symbolical power conferred by the particular context of the cure, the ritual in which it is prescribed, the ways of administration and the form or attitude of the person administrating it.

A common practise of Spanish origin, is to expose the plants to the "*sereno"* or dew and harvest them at sunrise on "Holy days" (generally Good Friday), after which they become "holy", an attribute no doubt connected with their power and, in turn, with their efficacy. References to uneven numbers are also frequent, especially 3 and 7, for the number of parts, doses and times of administration.

Traditional medicine and ritualistic cures also include the symbolic value of the curing places and elements. Hence, it is common to use "cepacaballo" (*Xanthium spinosum*) thorns placed in the shape of a cross to cure warts; others suggest carrying out this cure at crossroads, places which symbolically represent meeting points and encounters with the numinous.

Curing parasites combines the ingestion of plant species, especially garlic (*Allium sativum*), "zapallo" (squash) seeds (*Cucurbita* spp.), sugar (*Saccharum officinarum*), and the cure by words that is performed in a decreasing numeric countdown (for example, from 100 to 0), which metaphorically accompanies the progressive disappearance of the parasites, one by one, until being cured.

Likewise, healing by words is generally used when treating "*nervios*", toothaches, burns, warts, drunkenness and parasites, and is practised in the presence of the patient or at a distance. The cure for "*empacho", "pata de cabra",* "*culebrilla"* and liver disorders require, other than plants, a ritualistic cure which combines healing by words, measuring with a red tape, "*tirar el cuerito*" (pulling the skin), rubbings with ashes and saying prayers, or applications of Indian ink on the patient's skin**.**

Another common practice used in the area for treating toothaches or umbilical hernias in children, is the "*cura por el rastro*" (healing by a person's tracks). The procedure, which must be performed during a waning moon, involves drawing the perimeter of the patient's foot on the ground, or else in the bark of fig (*Ficus carica*) or "*chañar"* (*Geoffroea decorticans*) trees, cutting out the silhouette and leaving it to dry in a dark place.

On the other hand, the prevention of evils and harms uses plants that are generally not autochthonous and are acquired in herbariums and holy shops of the area, although in some cases they are grown at home like "ruda" (rue- *Ruta* spp.), "romero" (rosemary -*Rosmarinus officinalis*) and laurel (*Laurus nobilis*). Also used for this purpose are "palo santo" (*Bulnesia sarmientoi*), "cachiyuyo" (*Atriplex montevidensis*), garlic (*Allium sativum*), lemon (*Citrus limon*) and orange (*Citrus cinensis*). It is also common to incense with coffee (*Coffea arabiga*) and sugar (*Saccharum officinarum*) to prevent the entrance of bad spirits. Other preventative measures use and manipulate Catholic symbols and elements like holy water and plants like the olive tree (*Olea europaea*) or palm tree.

Apart from the diagnosis with water and oil, previously mentioned for its therapeutic value, *"ojeaduras"* (evil eyes), *"daños"* (harms) and "*males"* (evils) require prayers involving the sick child's name. Also frequent is the use of Christian symbols, like making the sign of the cross on the temples and forehead while imposing their hands and evocating the healing power of God (Holy Trinity, Jesus Christ), canonized saints (like Saint George) or popular canonizations (Pancho Sierra, Gauchito Gil). When healing by words is not enough

to treat *"ojeaduras"*, infusions of "guayacán" (*Porlieria microphylla*) leaves or rubbings with "alcanfor" (camphor- *Artemisia alba*) are also used.

II. Peasant Medicinal Plants

Characterization of Plant Pharmacopoeia

The catalogue of plants used in the peasant medicine of the Sierras de Córdoba analyzed in this review includes 1366 medicinal uses corresponding to 362 species, native and exotic, belonging to 91 botanical families. The complete detail of applications and uses can be found in each of the specific articles. **Table 1** presents a list of medicinal applications of the species with the greatest consensus among the regions and authors considered in this work.

Regarding the diversity of uses, **Figure 2** lists 20 species with the highest number of medicinal applications. About 70% of these species are native; the remaining 30% are either introduced cultivated species (15%) or adventitious species (15%). In first place, with more than 25 different uses, is "ruda" (rue- *Ruta chalepensis*), a cultivated or sometimes adventitious species; second is a native species, "contrayerba" (*Trixis divaricata* subsp. *discolor*). The use of rue is widespread among the *criollo* traditional medicine ambit of this region as well as in other areas of the country (Di Lullo, 1944; Arenas & Galafassi, 1994; Idoyaga Molina, 2000a,b). In both cases, the informers generally attribute special powers to these plants for treating any kind of imbalance, regardless of its origin (organic or social). Therefore, we understand that the number of uses for rue and "contrayerba" and their therapeutic capacity for a wide scope of afflictions constitutes an expression of their power in the sacred sense and not only to their pharmacological potential (Idoyaga Molina, 2000b)[4]; in turn, this evidences the need to approach these quantitative elements with an interpretative perspective. Other important native species (**Figure 3)** are bushes or trees like the "espinillo" (*Acacia caven* var. *caven*), "sombra de toro" (*Jodina rhombifolia*); aromatic bushes like "palo amarillo" (Whitebrush, *Aloysia gratissima* var. *gratissima*), "poleo" (*Lippia turbinata*) and "peperina" (*Minthostachys mollis*); non-aromatic bushes and sub-bushes like "malvavisco" (*Sphaeralcea cordobensis*), "quiebraarado" (*Heimia salicifolia*), "salvia blanca" (white sage, *Buddleja cordobensis*) and "*jarilla*"; herbs like "doradilla" (*Anemia tomentosa* var. *tomentosa*), "ortiga" (nettles- *Urtica urens*), "quimpe" (*Lepidium didymum*), "paico" (*Chenopodium ambrosioides*), among others.

Some of the introduced adventitious species (**Figure 4**) are "malva" (mallow- *M. parviflora, M. sylvestri*s) and "llantén" (plantain- *Plantago major*); among the cultivated species we find, other than rue, "altamisa" (tansy- *Tanacetum parthenium*), "romero" (rosemary- *Rosmarinus officinalis*) and "manzanilla" (chamomile- *Matricaria recutita*).

Depending on the habit, biological form and botanical origin of the species, **Figure 5** shows that most of the pharmacopoeia is composed of bushes and sub-bushes, followed by herbs and to a lesser degree trees, creepers, lianas and non-vascular forms. Introduced species

[4] Despite this, rue (*Ruta chalepensis*) has been greatly studied from a phytochemical point of view, and also for its therapeutic and pharmacological properties (Arteche García *et al.* 1998); however, "contrayerba" (*Trixis divaricata* subsp. *discolor*) has not been studied and there are only scarce references to its essential oils (Fester *et al*., 1961).

are especially relevant in the categories of trees and perennial herbs, and many are cultivated in gardens of the area.

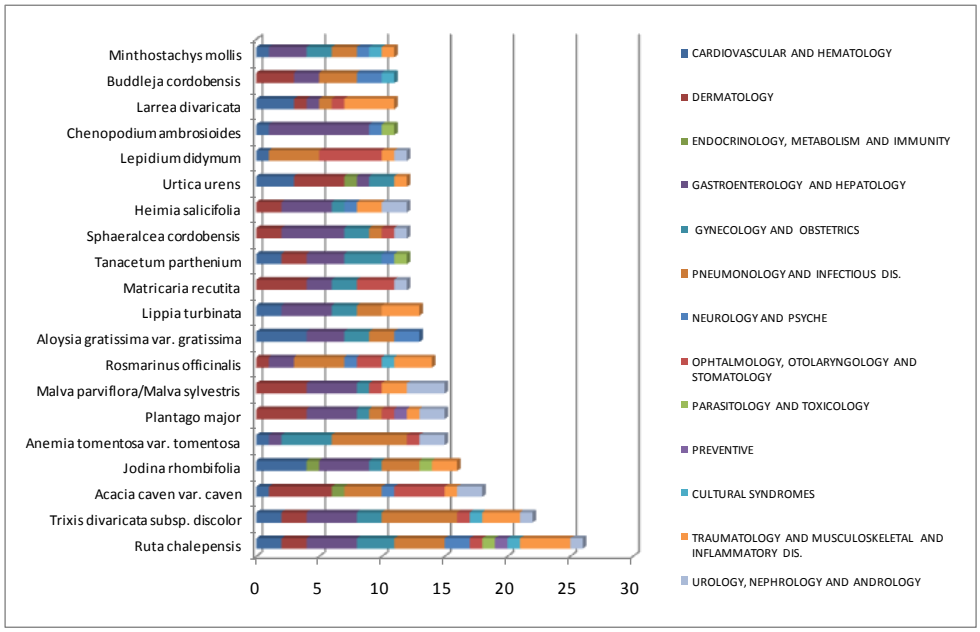

Figure 2. Percentage representation of the categories of medicinal applications of the species with the greatest number of uses.

Regarding their uses (**Figure 6**), most of the applications and medicinal species of the *serrano* pharmacopoeia are used for treating digestive and hepatic affections (23%), followed by skin (16%), respiratory (14%) and circulatory disorders (10%). Most of these applications use the aerial parts and leaves of the plants and therefore their harvest, if undertaken carefully, conveys no great ecological risks for the species; more impact is caused by the use of flowers, fruits or roots, although in general the use of these elements is far from the magnitude of the former. The most common preparations are decoctions and infusions in water, which are used as hot (*mate*, tea) or cold ("*aguapastos*" or "*aguapasta*") beverages, or else used for washes and baths. These forms of use and applications are especially destined to the treatment of gastrointestinal, hepatic, circulatory and dermatological ailments. Also frequent are external applications like topical uses, cataplasms and poultices used for treating respiratory affections and osteo-muscular disorders.

Figure 3. Native plants with the largest number of medicinal uses: a.-"aromito", *Acacia caven* var. *caven* b.- "doradilla", *Anemia tomentosa* var. *tomentosa* ; c.- "quiebra-arado", *Heimia salicifolia* ; d.- "contrayerba", *Trixis divaricata* subsp. *discolor* ; e.- "vira-vira", *Achyrocline satureioides* ; f.- "peperina", *Minthostachys mollis* ; g.- "sombra de toro", *Jodina rhombifolia* ; h.- "palo amarillo", *Aloysia gratissima var. gratissima.*

Table 1. Medicinal uses of the plants most frequently mentioned in different areas of Córdoba: (1) North region, (2) Southwest region (Sierra de Comechingones), (3) Mountains and valleys of the South region, (4) Central-West region.

Botanical taxon	Local names	Botanical Family	Indication or Ascribed therapeutic effect	Plant part used	Preparation, prescription and recipies	Areas of Córdoba
Acacia aroma Gillies ex Hook. & Arn.	tusca	FABACEAE	Skin diseases, disinfectant	Aerial part	Decoction, used to wash the affected area	(1), (3), (4)
Achyrocline spp. (*A. tomentosa* Rusby & *A. satureioides* (Lam.) DC.	marcela, vira vira	ASTERACEAE	Digestive and eupeptic.	Flowers, Aerial part	Infusion or decoction, drunk as tea	(1), (2), (3)
Baccharis articulata (Lam.) Pers.	carqueja, carquejilla	ASTERACEAE	Liver ailments, digestive	Aerial part	Decoction, drunk as tea	(1), (2), (3)
Celtis ehrenbergiana (Klotzch) Liebm.	tala	CELTIDACEAE	Digestive	Leaves	Infusion or decoction, drunk as tea	(1), (2), (3), (4)
Chenopodium ambrosioides L.	paico, paico macho	CHENOPODIACEAE	Digestive	Leaves	Infusion or decoction, drunk as tea	(1), (2), (3)
Commelina erecta L.	Santa Lucía	COMMELINACEAE	Ophtalmic	Flowers (mucilage)	Collyrium, direct application	(2), (3), (4)
Equisetum giganteum L.	cola de caballo	EQUISETACEAE	Diuretic, renal affections	Aerial part	Infusion or decoction, drunk as tea	(1), (2), (3)
Geoffroea decorticans (Gillies ex Hook. & Arn.) Burkart var. *decorticans*	chañar	FABACEAE	Bronchial diseases, antitusive and descongestive,	Bark	Decoction or infusion with or without sugar, drunk as tea or as a syrup	(1), (3), (4)

Table 1. (Continued)

Huperzia saururus (Lam.) Trevis.	cola de quirquincho	LYCOPODIACEAE	Aphrodisiac	Aerial Part	Infusion or decoction, drunk as tea	(1), (2), (3)
Minthostachys mollis (Kunth) Griseb.	peperina	LAMIACEAE	Digestive	Aerial part	Infusion or decoction, drunk as tea or with "mate"	(1), (2), (3), (4)
Passiflora caerulea L.	pasionaria	PASSIFLORACEAE	For treating anxiety and irritability; sedative	Leaf	Infusion or decoction, drunk as tea	(2), (3), (4)
Schinus areira L.	aguaribay	ANACARDIACEAE	Liver ailments, digestive	Leaves	Infusion or decoction, drunk as tea	(1), (2), (3)
Senna corymbosa (Lam.) H.S. Irwin & Barneby	sen del campo	FABACEAE	Drastic, purgative	Aerial part	Infusion or decoction, drunk as tea	(1), (2), (3)
Ruta chalepensis L.	ruda, ruda macho	RUTACEAE	Liver ailments, digestive	Aerial part	Infusion, drunk as tea	(1), (3), (4)
Scoparia montevidensis (Spreng.) R.E. Fr.	canchalagua	SCROPHULARIACEAE	Liver ailments	Aerial part	Infusion or decoction, drunk as tea	(1), (2), (3)
Aloysia citriodora Palau	cedrón	VERBENACEAE	For palpitations and cardiac diseases; for anxiety and irritability; sedative	Leaf	Infusion or decoction, drunk as tea	(1), (3), (4)
Lippia turbinata Griseb.	poleo	VERBENACEAE	Digestive	Aerial part	Infusion or decoction, drunk as tea	(1), (2), (3)

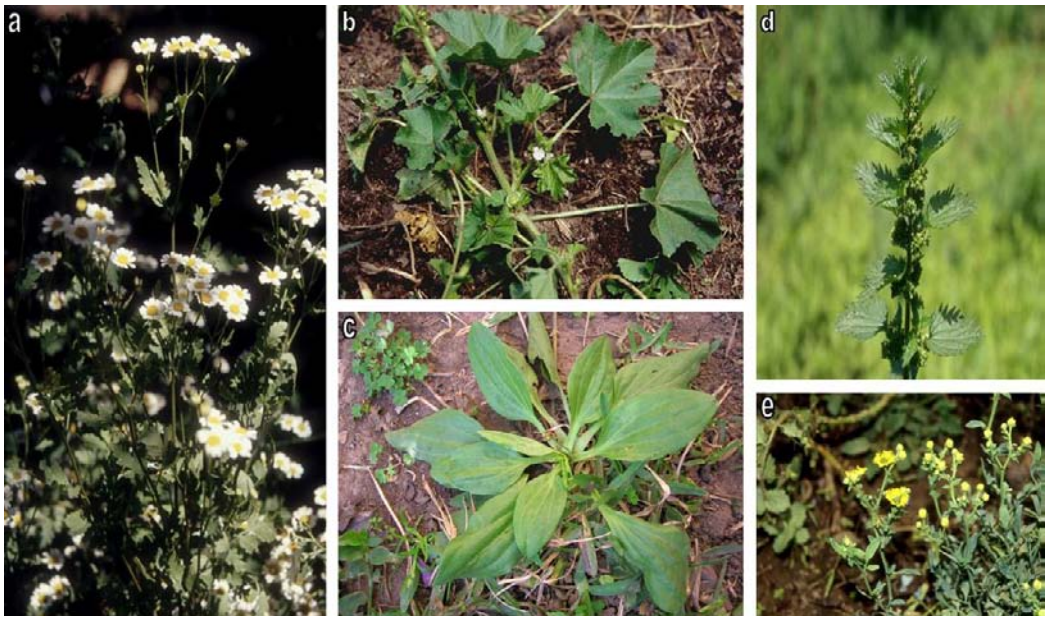

Figure 4. Introduced species (cultivated and adventitious) with the largest number of medicinal uses: a.- "altamisa", *Tanacetum parthenium*; b.- "malva", *Malva parviflora*; c.- "llantén", *Plantago major*; d.- "ortiga", *Urtica urens*; e.-"ruda", *Ruta chalepensis*

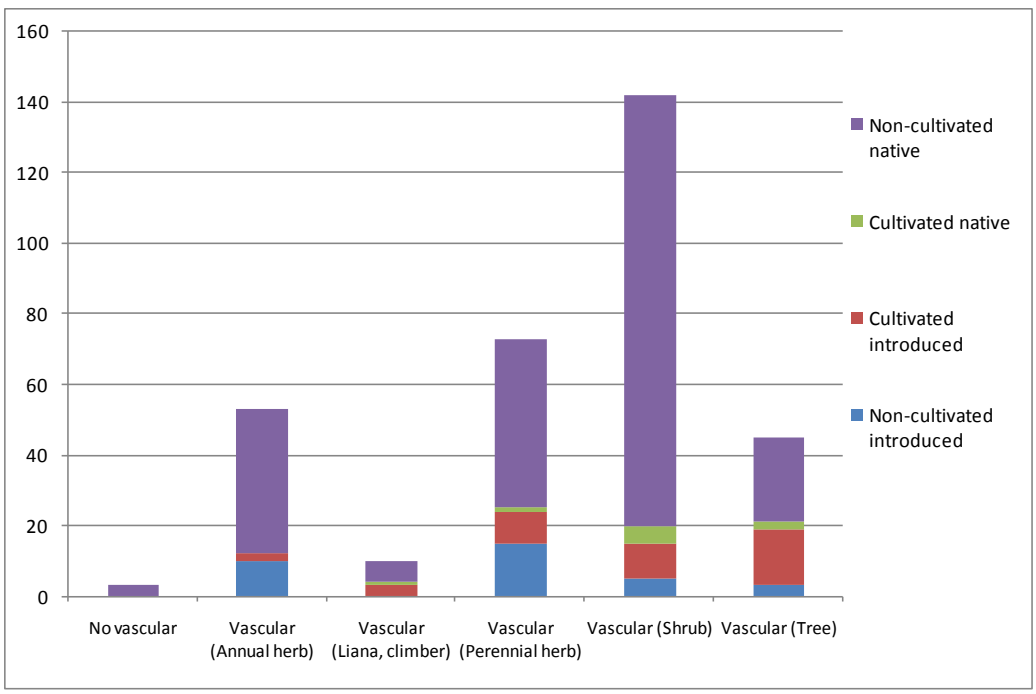

Figure 5. Number of medicinal species grouped by biological category and habit.

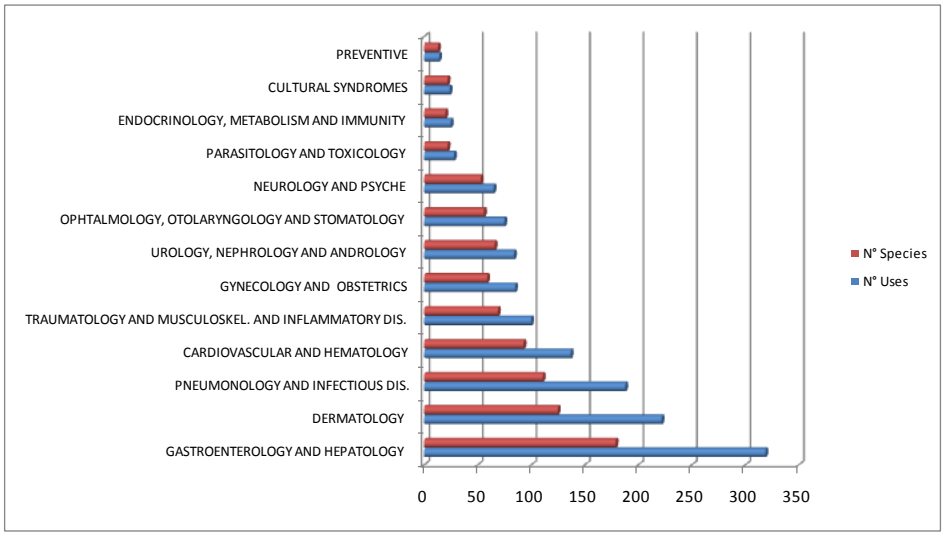

Figure 6. Number of species and uses for each category of medicinal applications.

Considering the consensus between informers regarding the medicinal applications in different health specialties, **Table 2** shows that the greatest flow of joint information and the most defined criteria belongs to gastrointestinal (Fic= 0.54), skin (=0.42), respiratory and infectious (Fic=0.42) diseases. On the contrary, the lowest values of consensus belong to the species used for the treatment of endocrine-metabolic diseases (Fic=0.23) and cultural syndromes (Fic=0.19): the first case shows a more random selection of species, while the second reflects an exclusive cultural knowledge restricted to only a few individuals.

Legislation and Control

Throughout this review we have seen how the efficiency, and hence therapeutic value, of a plant for the peasant culture is interpreted not only from a pharmacological aspect but also from a symbolical point of view, as in the case of species attributed special powers. Therefore, from a strictly pragmatic and practical point of view, knowing the criteria a community uses to select its plants for treating diseases and ethnobotanical medical studies is a good basis on which to orient the search for new useful substances, an aspect with important ethical questionability regarding the usufruct of traditional knowledge (Arenas, 1996).

Many studies describe the chemical composition and active substances of some of the native and introduced plant species we have mentioned. Some of the botanical collaborations mentioned in the introduction review the phytochemical aspects, such as the work of Barboza *et al*. (2006) and the compilations made by Alonso (1998, 2004) and Alonso & Desmarchelier (2006). The specific articles of some research groups on the native plants of Córdoba are also important references, especially certain local studies of international relevance (Agnese & Cabrera, 1996; Cabrera & Juliani, 1981; Guglielmone *et al*., 2005; Juliani *et al*., 2002; Ortega, 2002; Ortega *et al*., 2004, 2006; Zygadlo & Guzman, 1991, 1993; Zygadlo *et al*., 1994, 1996; Bongiovanni *et al*., 2006, 2008; Soria *et al*., 2008; Zunino *et al*., 2003, and others). There are also important online databases, for example NAPRALERT

(Natural Product Alert), providing information on the chemical composition and properties of many of the species mentioned here, which is why we will not enter into any chemical details. It is clear that the phytochemical and pharmacological investigations of native species has allowed, in more than one occasion, to corroborate the validity of peasant empirical knowledge established on the basis of trial and error or other means. These studies reveal the presence of species with pharmacologically active substances, evidencing the harmlessness of some species (that does not necessarily imply it is inefficient within a symbolical aspect) and, in some cases, the toxicity of others. Regarding the latter, it is noticeable that none of the inhabitants and doctors we interviewed mentioned any case of intoxication due to the use of medicinal plants. Although probably influenced by their academic formation rather than by an empirical confirmation of documented cases of intoxications, it is clear that biomedical professionals resist prescribing medicinal herbs. Therefore, and in order to prevent the indiscriminative use of plants with active substances, we consider it is necessary to point out the species in the catalogue that require special caution in their management or prescription. We consider that this provides useful criteria to select species for conservational means or to promote or discourage their cultivation in gardens, vegetable gardens and live pharmacies, and that, when used properly, would greatly benefit the health of peasant communities and the development of primary health care strategies with therapeutic complements.

Even when there is still no legislation for most of the applications of species used in the traditional medicine of Córdoba, some are specifically mentioned in publications and regulatory legislations currently in force in Argentina, under the regulatory ambit of the Argentine National Pharmacopoeia (Farmacopea Nacional Argentina - FNA) and National Drug Institute (Instituto Nacional de Medicamentos - INAME-ANMAT) (Agnese *et al*., 2002). **Table 3** shows the native and introduced species commonly used in the traditional medicine of the Sierras de Córdoba that are systematically codified for our country in editions 1º to 6º of the FNA and in legislative dispositions Nº 2673/99 and 1788/2000 of the INAME-ANMAT on phytotherapeutics, both in the positive and negative list of drugs, authorized and non-authorized respectively.

As can be observed, the list of codified species is very scarce compared to the diversity of plants of the highland pharmacopoeia. In a few years, new pharmacological, pharmacobotanical and phytochemical studies will extend the list of authorised and codified species, and with it the National Argentine pharmacopoeia, thus regulating new uses which, although common among the peasants, are not taken into account by biomedical professionals and, to an even less extent, in primary health care practices. Hence, it will be very valuable to have pharmacobotanical studies on the plants of our province and the rest of Argentina as those carried-out by local researchers (Filippa & Ariza Espinar, 1993; Filippa, 2004; Bonzani *et al*., 1997; 2003a,b; Barboza *et al*., 2001; Ariza Espinar & Bonzani, 1992; Luján & Barboza, 1999; Luján *et al*., 2001, 2004) and quality control measures on the products used and/or commercialized.

III. Extraction, Use, Comercialization and Conservation of Medicinal Plants

[3] Medicinal plants not only have an important role in traditional health systems, but also in international herb and pharmaceutical markets. With the increased demand for natural phytotherapeutics and pharmaceuticals, the local cultures and biological resources have become increasingly susceptible to the pressure of market economies. In some cases, the ability to provision is becoming critical, as evidenced by the increasing distances the natives have to travel to collect their medicines and the documentation of over-exploited commercial species (Martínez, 2005a).

Although one-fourth of the 250,000 medicinal species currently known are found in Latin–America (Elisabetsky & Costa-Campos 1996), there is scarce information concerning endangered species for the region (Lucas & Synge 1978; Davies *et al*. 1986). A partial list of endangered and threatened plants for Argentina can be found in De la Sota (1977), Cabrera (1977), Noher de Halac *et al*. (1986), Delucchi & Correa (1992), and Vischi *et al*. (2004), but the records about medicinal flora is scarce. In the province of Córdoba, the available information on harvesting and commercializing medicinal plants comes mainly from Traslasierras (to the west of Córdoba) (Lagrotteria *et al*., 1986, 1987a,b; Rodríguez *et al*., 1992; Lagrotteria & Affolter, 1999), a region from which great volumes of medicinal plants are collected, extracted, stored and commercialized. Although of much lesser importance, the collection and commercialization in the Department of Santa María (southwest Córdoba) involves 64 medicinal species, many of which are wild native plants growing in the area (Martínez, 2005a). In order to estimate the impact of these practices, we developed a quantitative method for evaluating the conservation priorities of species used in popular medicine (Martínez *et al*., 2006). In this study, qualitative attributes were surveyed by the knowledge and perception of local communities and we ranked the species according to their index of conservation priority (ICP), which considers the following data: harvest, perceived abundance, propagation method, origin and commercial demand of the species in the area.

Following these criteria and others proposed by authors of similar studies for different regions of the province of Córdoba, **Table 4** and **Figure 7** shows a list of medicinal species prioritized in terms of conservation. The prominent pressure of extraction, non-sustainable harvest and commercialization practices, restriction of its distribution (endemism and/or species with restricted distributions), or the combination of all these factors, together with the absence of a legal framework and other environmental problems like habitat degradation, leads to a loss of genes threatening the continuity of some of the native medicinal species of our hills.

Seeking the sustainable use and management of medicinal flora, the Chiang Mai declaration (WHO-UICN-WWF, 1993) indicates that, among the conservation strategies the propagation of wild autochthonous plants in cultivation systems requires studies on the variability, germination and propagation of these species, among other aspects.

Table 2. Category of medicinal uses according to their decreasing values of Informant Consensus Factor (F_{ic})

Category of medicinal uses (group of illness)	Number of taxa (n_t)	% taxa	Nº Uses (n_u)	% Uses	Number of use-reports (n_{ur})	% use-reports	Number uses/ Number taxa	Factor F_{ic} = (n_{ur} - n_t)/ (n_{ur} - 1)	Species with higher frequency of citations
GASTROENTEROLOGY AND HEPATOLOGY	180	49,05	319	23,35	392	25,45	1,77	0,54	Chenopodium ambrosioides L., Schinus areira L., Lippia turbinata Griseb., Sphaeralcea cordobensis Krapov., Ruta chalepensis L., Porlieria microphylla (Baill.) Descole, O´Donell & Lourteig, Celtis ehrenbergiana (Klotzch) Liebm., Jodina rhombifolia (Hook. & Arn.) Reissek, Artemisia douglasiana Besser, Rosmarinus officinalis L., Minthostachys mollis (Kunth) Griseb., Aloysia gratissima (Gillies & Hook. ex Hook.) Tronc. var.gratissima, Mentha x rotundifolia (L.) Huds./ Mentha longifolia , Prunus persica (L.) Batsch, Marrubium vulgare L., Trixis divaricata (Kunth) Spreng. subsp. discolor (D. Don) Katinas, Plantago major L., Artemisia absinthium L.
DERMATOLOGY	126	34,33	221	16,18	235	15,26	1,75	0,47	Aloe spp., Acacia aroma Gillies ex Hook. & Arn., Acacia caven (Molina) Molina var. caven, Nicotiana glauca Graham, Chenopodium aff. murale L., Chenopodium album L., Gaillardia megapotamica (Spreng.) Baker var. scabiosoides (Arn. ex DC.) Baker, Plantago major L., Malva parviflora L.. Malva sylvestris L., Matricaria recutita L., Rumex crispus L., Sphaeralcea cordobensis Krapov., Urtica urens L.

Table 2. (Continued)

PNEUMONOLOGY AND INFECTIOUS DISEASES	111	30,25	188	13,76	207	13,44	1,69	0,47	Geoffroea decorticans (Gillies ex Hook. & Arn.) Burkart var. decorticans, Anemia tomentosa (Savigny) Sw. var. tometosa, Eriobotrya japonica (Thunb.) Lindl., Trixis divaricata (Kunth) Spreng. subsp. discolor (D. Don) Katinas, Achyrocline satureioides (Lam.) DC., Croton subpanossus Mull. Arg., Rosmarinus officinalis L., Buddleja cordobensis Griseb., Lepidium didymum L., Eucalyptus cinerea F. Muell. ex Benth., Usnea sp., Acacia caven (Molina) Molina var. caven, Ruta chalepensis L., Laurus nobilis L., Jodina rhombifolia (Hook. & Arn.) Reissek
CARDIOVASCULAR AND HEMATOLOGY	93	25,34	137	10,03	158	10,26	1,47	0,41	Jungia polita Griseb., Cuphea glutinosa Cham. & Schltdl. Hypericum connatum Lam., Jodina rhombifolia (Hook. & Arn.) Reissek. Ligaria cuneifolia (Ruiz & Pav.) Tiegh., Passiflora caerulea L., Aloysia gratissima (Gillies & Hook. ex Hook.) Tronc. var.gratissima, Equisetum giganteum L., Urtica urens L., Acanthospermum australe (Loefl.) Kuntze, Acanthospermum hispidum DC., Aloysia citriodora Palau, Larrea divaricata Cav.
TRAUMATOLOGY AND MUSCULOSKELETAL AND INFLAMMATORY DISEASES	68	18,53	99	7,25	109	7,08	1,46	0,38	Larrea divaricata Cav., Ruta chalepensis L., Ephedra triandra Tul. Emend. J.H.Hunz.
OBSTETRICS AND GYNECOLOGY	59	16,08	85	6,22	91	5,91	1,44	0,36	Anemia tomentosa (Savigny) Sw. var. tometosa, Tanacetum parthenium (L.) Sch. Bip., Margyricarpus pinnatus (Lam.) Kuntze, Tripodanthus flagellaris (Cham. & Schltdl.) Tiegh, Ruta chalepensis L.

Table 2. (Continued)

Ophtalmology, Otolaryngology And Stomatology	55	14,99	75	5,49	81	5,26	1,36	0,33	Acacia caven (Molina) Molina var. caven, Lepidium didymum L., Usnea sp., Commelina erecta L.
Parasitology and Toxicology	22	5,99	28	2,05	32	2,08	1,27	0,32	Tagetes minuta L., Melia azederach L.,Artemisia absinthium L., Chenopodium ambrosioides L., Schkuhria pinnata (Lam.) Kuntze ex Thell.
Urology, nephrology and andrology	66	17,98	84	6,15	96	6,23	1,27	0,32	Equisetum giganteum L., Huperzia saururus (Lam.) Trevis., Anemia tomentosa (Savigny) Sw. var. tometosa, Malva sylvestris L., Xanthium spinosum L. var. spinosum, Plantago major L., Malva parviflora L.
Preventative	13	3,54	14	1,02	18	1,17	1,08	0,29	Lithrea molleoides (Vell.) Engl., Achyrocline satureioides (Lam.) DC., Hedeoma multiflora Benth., Tagetes minuta L., Psidium salutare (Kunth) O. Berg
Neurology and Psyche	53	14,44	65	4,76	69	4,48	1,23	0,24	Passiflora caerulea L., Huperzia saururus (Lam.)
Endocrinology, Metabolism and Immunity	21	5,72	26	1,90	27	1,75	1,24	0,23	Bauhinia forficata Link subsp. pruinosa (Vogel) Fortunato & Wunderlin, Huperzia saururus (Lam.) Trevis., Taraxacum officinale G. Weber ex F.H. Wigg., Schkuhria pinnata (Lam.) Kuntze ex Thell.
Cultural Syndromes	22	5,99	24	1,76	25	1,62	1,09	0,13	Porlieria microphylla (Baill.) Descole, O´Donell & Lourteig, Artemisia alba Turra, Minthostachys mollis (Kunth) Griseb.
Total	(362)	-	1366	100,00	1540	100,00			

Table 3. Codification of medicinal plant uses according to the national official regulations of Argentina.

Genus and species	Recognized therapeutic action	Codified by the Argentine National Pharmacopoeia (F.N.A.)	Codified in the positive list of drugs approved for phytotherapy (INAME-ANMAT Disp. Regl. 2673/99, Anexo III)	Codified in the negative list of drugs not approved for phytotherapy (INAME-ANMAT Disp. Regl. 1788/2000)
Carqueja, carquejilla *Baccharis crispa*	Hepatic	F.N.A. 6º Ed.	--	--
Carquejilla, carqueja *Baccharis articulata*	Hepatic	F.N.A. 6º Ed.	--	--
Cedrón *Aloysia citriodora*	Sedative	--	YES (Leaves)	--
Paico *Chenopodium ambrosioides*	Digestive, stomachic	--	--	YES (prohibited)
Pasionaria- *Passiflora caerulea*	Sedative	--	YES (Leaves)	--
Melisa o toronjil -*Melissa officinalis*	Sedative	F.N.A. 4º Ed.	--	--
Cola de caballo *Equisetum giganteum*	Diuretic	F.N.A. 6º Ed.	YES (Aerial part)	--
Tasi o doca *Morrenia brachystephana*	Galactogogus	F.N.A. 1º Ed.	--	--
Vira vira o marcela *Achyrocline satureioides*	Expectorant	--	YES (Flowers and leves)	--
Granada *Punica granatum*	Antidiarrhoeal	F.N.A. 3º Ed.	--	--
Malva *Malva sylvestris*	Antihemorrhoidal	F.N.A. 4º Ed.	--	--
Eucaliptos *Eucalyptus* spp.	Pectoral	F.N.A. 6º Ed.	--	--
Poleo *Lippia turbinata*	Digestive, stomachic	F.N.A. 6º Ed.	YES (Aerial part)	--
Nogal *Juglans regia*	To treat seborrhoea, Antidandruff	F.N.A. 2º Ed.	--	--
Tilo *Tilia* spp.	Sedative	F.N.A. 6º Ed.	--	--
Cola de gama *Heliotropium curassavicum*	Hypocholesterolemiant	--	--	YES (prohibited)

Figure 7. Native medicinal plants from Sierras de Córdoba with conservation priorities: a) "poleo" *Lippia turbinata*; b) "carqueja" *Baccharis articulata*; c) "carquejilla" *Baccharis crispa*; d) "cabotoril" *Hypericum connatum*; e) ""pasionaria" *Passiflora caerulea*; f) "peperina" *Minthostachys mollis*; g) "culandrillo" *Adiantum* sp.; h) "cola de caballo" *Equisetum giganteum*; i) "cola de quirquincho" *Huperzia saururus*; j) "barba de piedra" *Usnea* sp.

Table 4. Native medicinal plants from the Sierras de Córdoba with conservation priorities according to different criteria (ecological abundance, extraction, and commercial demand, frequency of use, etc.) presented by: (1) De La Sota (1977); Cabrera (1977); (2) Noher de Halac *et al.* (1986); (3) Lagrotteria and Affolter (1999); (4) Martínez, (2003); Martínez *et al.*, (2006); (5) Goleniowski *et al.*, (2006); (6) Bocco *et al.* (1997, 1993), (7) Vischi *et al.* (2004) -Species with three or more references from different authors are highlighted in bold

Species (Family)	Local name	References
Achyrocline satureioides (Lam.) DC. (Asteraceae)	Marcela Vira-vira	(2), (3), (4), (6)
Adiantum spp. (A. raddianum C. Presl/ A. thalictroides Willd. ex Schltdl./ A. lorentzii Hieron.) (Adiantaceae)	Culandrillos	(1), (2), (3), (4), (5)
Aloysia gratissima (Gillies and Hook.) Tronc. (Verbenaceae)	Palo amarillo Azahar del campo	(2), (3), (4)

Table 4. (Continued)

Species (Family)	Local name	References
Anemia tomentosa var. tomentosa (Savigny) Sw. (Anemiaceae)	Doradilla	(2), (3), (4)
Aristolochia stuckertii Speg. (Aristolochiaceae)	Charrúa	(5)
Aspidosperma quebracho-blanco Schlecht (Apocynaceae)	Quebracho blanco	(5)
Baccharis articulata (Lam.) Pers. (Asteraceae)	Carquejilla Carqueja	(2), (3), (4), (6)
Baccharis crispa Spreng. (Asteraceae)	Carqueja carquejilla	(2), (3), (4), (6)
Buddleja cordobensis Griseb. (Buddlejaceae)	Salvia blanca Yerba del águila	(7)
Canna glauca L. (Cannaceae)	Achira	(5)
Capsicum chacoense Hunz. (Solanaceae)	Ají del monte	(5)
Croton subpannosus Mull. Arg. ex Griseb. (Sin: Julocroton subpannosus var. subpannosus) (Euphorbiaceae)	Pulmonaria	(4)
Cuphea glutinosa Cham. and Schltdl. (Lythraceae)	sanguinaria	(4)
Chenopodium ambrosioides L. (Chenopodiaceae)	Paico	(3), (4)
Species (Family)	Local name	References
Ephedra americana Humb. & Bonpl. (Ephedraceae)	Tramontana colorada	(3)
Ephedra triandra Tul. emend. J.H. Hunz. (Ephedraceae)	Tramontana, pico de loro	(3)
Equisetum giganteum L. (Equisetaceae)	Cola de caballo	(2), (3), (4), (6)
Gaillardia megapotamica var. scabiosoides (Arn. ex DC.) Baker (Asteraceae)	topasaire	(4)
Geoffroea decorticans (Gillies ex Hook. and Arn.) Burkart (Fabaceae)	Chañar	(4)
Hedeoma multiflora Benth. (Lamiaceae)	Tomillo de la sierra	(2), (3), (4), (5), (6)
Huperzia saururus (Lam.) Trevis. (Lycopodiaceae)	Cola de quirquincho	(1), (2), (3), (4), (5)
Hypericum connatum Lam. (Clusiaceae)	Cabotoril cabotorilo	(2), (3), (4)

Killinga odorata Vahl (Cyperaceae)	Capií-catí	(5)
Lippia turbinata Griseb. (Verbenaceae)	Poleo	(2), (3), (4), (6)
Jungia polita (Asteraceae)	Zarzaparrilla	(4), (5)
Lycopodium clavatum L. (Lycopodiaceae)	Pilliján	(5)
Margyricarpus pinnatus (Lam.) Kuntze. (Rosaceae)	Yerba de la perdiz	(3), (4)
Minthostachys mollis Griseb. (Lamiaceae)	Peperina	(2), (3), (4), (6)
Passiflora caerulea L. (Passifloraceae)	Pasionaria	(2), (3), (4)
Phacelia pinnatifida Griseb. ex Wedd. (Hydrophyllaceae)	Yerba meona	(4)
Porophyllum obscurum (Spreng.) DC. (Asteraceae)	Yerba del venado	(4), (5)
Scoparia montevidensis (Spreng.) R.E. Fr. (Scrophulariacae)	Canchalagua	(3), (4)
Solanum sisymbriifolium Lam. (Solanaceae)	Espina colorada	(4)
Trixis divaricata subsp. discolor (D. Don) Katinas (Asteraceae)	contrayerba	(4)
Usnea sp. (Usneaceae)	Barba de piedra	(2), (3), (4)

Experiments of this sort have been initiated in our country with species belonging to the local pharmacopoeia of the Córdoba hills. Among them we must mention, due to their degree of development, the work of Ojeda (2004) and Ojeda *et al*. (2000a,b; 2001; 2004; 2006 and others) that comprise a true integral "domestication" plan for "peperina" (*Minthostachys mollis*) plants. These studies characterized the growing environments of the species and specimens were evaluated *in situ* (Ojeda *et al*., 2001). Also, seeds were collected from different sources (populations) and germination tests were carried out in nursery gardens and in the field (Ojeda, 2004). Field tests were performed to determine culture management, evaluating morphological, phenologic, cytologic and biochemical characters (Ojeda *et al*., 2000b; Ordóñez *et al*., 2002; Ojeda *et al*., 2004; Goirán *et al.*, 2006). The results obtained to the present day have enabled the selection of prime materials. A further characterization will eventually offer the general public an adapted culture material, with an appropriate production capacity for commercial use. In this sense it is important to note that this is the first cultivar of a native aromatic species: the "peperina" cultivar (Registro Nacional de Cultivares, phytogenetic creation of *Peperina Minthostachys mollis* (Kunth) Griseb., named Champaquí FCA, obtained by the Universidad Nacional de Córdoba – Facultad de Ciencias Agropecuarias), generated by research work aimed to improve the species for its cultivation. While continuing it its work on "peperina", the group is also studying other species like

"carquejas" (*Baccharis spp*.), "suico" (*Tagetes minuta*), "incayuyo" (*Lippia integrifolia*) and "tomillo serrano" (*Hedeoma multiflora*), furthering their characterization, analysing their inter-population variability, composition and essential oil properties, among other aspects (Ordóñez *et al*., 2006; Massuh, 2007). There are also similar studies on the germination, multiplication and micropropagation of "pasionaria" (*Passiflora caerluea*) (Martínez, 2003; Martínez *et al*., 2007), "tomillo serrano" (*Hedeoma multiflorum*) (Lagrotteria *et al*., 1993; Brunetti *et al*., 2007; Vázquez *et al*., 2007), "poleo" (*Lippia turbinata*) (Ortiz *et al*., 2007), "paico" (*Chenopodium ambrosioides*) (Rolando *et al.,* 1998), "jarilla" (Larrea divaricata) (Palacio *et al*., 2006), "marcela" (*Achyrocline satureioides*) (Nóbile *et al*., 1999) and "melisa" (*Melisa officinalis*) (Lloret *et al*., 2007). However, considering the extensive list of medicinal species, these propagation studies are still insufficient and an important number of plants with relevance in the highland peasant ethnomedicine remain to be studied from this agronomical perspective.

From an ethnobotanical point of view, the native bushes of the *Aloysia* genus (Verbenaceae) like "té de burro" (*Aloysia polystachya*) and "cedrón" (lemon verbena-*Aloysia citriodora*), ferns like "cola de caballo" (*Equisetum giganteum*) and "doradilla" (*Anemia tomentosa*), and to a lesser degree other species like "cabotoril" (*Hypericum connatum*), "palo amarillo" (*Aloysia gratissima*), "zarzaparrilla" (*Jungia polita*), as well as most of the species previously mentioned, are promising for these types of studies because despite growing spontaneously, they are occasionally grown in gardens and peridomestic areas, evidencing an incipient and gradual process of *in situ* local "domestication" by the highland inhabitants.

Finally, we can't forget the close relation between conservation and education, as the survival and circulation of the traditional knowledge of these folk cultures, including their medicine, is deeply connected to the conservation of the resources at stake. In this sense it is necessary to revitalize the efforts promoting a regionalized education, encouraging the reinstatement of this knowledge in formal and informal educational ambits, as well as in others promoting the conservation of natural plant resources. In a preliminary way, we have presented some publications regarding this topic (Martínez, 2002; Martínez *et al*., 2002, 2003), trying to include this problematic in the rural classrooms of this area. Several extension studies developed in schools and with the general community have been supported by the Secretaria de Extensión of the Universidad de Córdoba and other institutions (Ojeda *et al*. 2000a; 2006; Ojeda 2008), giving place to sustainable management experiences and regional enterprises, as well as the development of audiovisual and multimedia materials on the topic that are currently in circulation within the communities (Goirán *et al*., 2006; Martínez *et al*., 2006; Martínez & Villalba, 2006; Equipo de Etnobiología, 2006).

Conclusion

This review shows that the traditional medicine of the Sierras de Córdoba is a deeply rooted cultural component, and its greatest expression is found in the ambit of home remedies and healers. With its distinctive aspects, and others shared with the traditional medicine of different regions of Argentina, the use of a vast number of natural remedies is a characteristic

feature of the *serrano* therapeutics. In this sense, the knowledge local people have on their natural environment is noticeable; furthermore, they are practically capable of self-satisfying their therapeutic needs. Thus, the more than 300 species and 1,300 medicinal uses ascertained by ethnobotanical studies clearly evidence their preference in using native bushes and herbs, harvesting wild plants as a privileged form of obtaining provisions. Likewise, and from an ethnobotnanical point of view we point out species like "barba de piedra" (*Usnea* spp.), "doradilla" (*Anemia tomentosa*), "cola de caballo" (*Equisetum giganteum*), "peperina" (*Mintosthachys mollis*), "pulmonaria" (*Croton subpannosus*), "pasionaria" (*Passiflora caerulea*), "contrayerba" (*Trixis divaricata* subsp. *discolor*), "zarzaparrilla" (*Jungia polita*), "canchalagua" (*Scoparia montevidensis*), "carquejas" (*Baccharis crispa, Baccahris articulata*), "cola de quirquincho" (*Huperzia saururus*), "tomillo" (*Hedeoma multiflora*), "poleo" (*Lippia turbinata*), "palo amarillo" (*Aloysia gratissima* var. *gratissima*), for their widespread use in different highland regions, the great value given to their medicinal properties and their local conservation preferences.

Even when many of the applications have been validated by vernacular practices, the contribution of science (by pharmacobotanical, phytochemical, pharmacological and/or agronomical studies), will encourage and/or disseminate new medicinal uses as a therapeutic complement to biomedicine or official medicine, particularly in the context of multiple medicines as those observed in these communities, with important suggestions for projects contemplating primary health care systems such as those promoted by WHO. Likewise, it will provide sustainable advise on the selection of species destined to *in situ* and *ex situ* conservation or the propagation of species in cultivation systems.

Finally, with this review we hope to enhance the circulation of local knowledge, and with it reinforce the identifying value it bears for the inhabitants of the Sierras de Córdoba.

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Chapter 3

Gastroprotective Triterpenoids: Pharmacological Mechanism

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Abstract

Gastric and duodenal ulcers affect a considerable amount of people in the world. Ulcer occurs when there is a disturbance of the normal equilibrium caused by either enhanced aggression or diminished mucosal resistance of gastrointestinal tract. Mucosa defends gastrointestinal tract of acid, pepsin, bile, leukocyte infiltration and external substances such as alcohol, caffeine, chilli or certain drugs such as NSAIDs. The defense mechanisms of the gastrointestinal mucosa mainly consist of functional, humoral and neuronal factors. Mucus alkaline secretion, mucosal microcirculation and motility act as functional factors, while prostaglandins and nitric oxide (NO) act as humoral factors, and capsaicin-sensitive sensory neurons (CPSN) act as neuronal factors. Several plants containing triterpenoids have been shown to possess anti-ulcer activity. The gastroprotective effects of triterpenoids have been studied on ethanol or NSAIDs-induced gastric injury models. These models induced impairment in the mucosal defense process with the consequent gastric damage. The principal mechanism of gastroprotection of triterpenoids has been reported by the activations of mucous membrane secretion instead of the inhibition of gastric acid secretion. Chemically, this gastroprotective effect has been referred to the presence of a hydroxyl group free or derivative at position C-3 for sterols and triterpenoids. Some pharmacological gastroprotective mechanisms for this kind of natural products has been attributed to the role of prostaglandins, nitric oxide (NO), sulfhydryl groups (-SH), and capsaicin-sensitive afferent neurons. Besides, recently leukocyte adherence, TNF-α and hydrogen sulfide has been implicated on mucosal defense mechanism, however it is unknown on triterpenoids gastroprotection.

1. Introduction

Gastric injury is present in people from both development and undeveloped countries. Stress and ingestion of irritants such as caffeine, alcohol, nicotine, and drugs like NSAIDs cause it and also highly spicy food. Endogenous irritants may induce also certain injury at the mucosa levels, for example acid, bile and pepsin.

In the searching for the best treatment against gastric damage, researches have found several mechanism of how this injury is induced. The understanding of how gastric injury occurs has helped to the development of new therapies. Many inflammatory mediators play an important role in gastric mucosa safety.

Drug treatment of peptic ulcer is targeted at either counteracting the aggressive factors or stimulating the mucosal defense. In spite of the progress in conventional chemistry and pharmacology in producing effective drugs, the plant kingdom might provide a useful source of new anti-ulcer compounds for development as pharmaceutical entities or, alternatively, as simple dietary adjuncts to existing therapies.

Natural products are a significant source of compounds with gastroprotective properties. Between them, stand out the triterpenes, who have showed this property, and for which the gastroprotective mechanism has been elucidated in certain way.

In this chapter is outlined the research performed on the properties, pharmacological mechanisms and chemical features of triterpenes with gastroprotective properties.

2. Peptic Ulcer

2.1. Definition

An ulcer is the disruption of the mucosal integrity of the stomach or duodenum leading to a local defect or excavation due to active inflammation [1]. Peptic ulcer is a chronic lesion in any portion of the gastrointestinal (GI) tract. This ulcer is extended throw the muscular layer of the mucosa to the submucosa or deeper. Duodenum and stomach are the most common sites on the GI tract were peptic ulcer could appear [2]. In developed countries, 10% of population develop ulcer at least one moment on their lives. In United States, 4 millions of people are affected by peptic acid disorders.

For a better understanding of how peptic ulcer is induced, it is necessary the knowledge of acid secretion and mucosal defense mechanisms.

2.2. Gastric Anatomy

The stomach can be divided into 3 areas (fundus, corpus and antrum). The gastric wall consists in mucosa, submucosa, muscularis layer and serose. The stomach area, as a gastric gland is divided in:

a) *Gastric cardia gland*, include less than 5 % of gastric gland area and contains mucus secreting cells and endocrine cells.
b) *Oxyntic gland area,* localized on the fundus and corpus; this part of the stomach contains parietal, chief, endocrine, and enterochromaffin cells. Oxyntic means "acid builder".
c) *Pyloric gland area* contains mucous and endocrine cells. This area is found in the antrum.

Parietal cells or *oxyntic cells* are one of the most important cells in the stomach. The resting or unstimulated parietal cell has cytoplasmic tubulovesicles and intracellular canalliculi containing short microvilli along its apical surface. On the membrane of the tubulovesicles are expressed H^+/K^+ ATPases, which bombs hydrogen ions throw the membrane in exchange with potassium ion.

Chief cells are found in the base of oxyntic glands and secret pepsinogen I and II contained in granules. The pepsinogen is activated to pepsin by luminal pH and it is inhibited for high pH (pH 6) such as in duodenum.

Endocrine cells are distributed between the epithelial cells of the gastric and antral glands. In the antral mucosa the endocrine cells produce gastrin. In the corpus region, this kind of cells secretes histamine.

2.3. Pathology

Peptic ulcer is a consequence of the disturbance between aggressive and protective factors in the mucosa. Gastric mucosa has a special barrier against noxious agents. It has been described that exogenous noxious stimulus contribute to gastrointestinal injury such as *Helicobacter pylori* [3], NSAIDs (non-steroidal anti-inflammatory drugs) [4, 5], nicotine [6], alcohol [7], chilli, caffeine and other irritant food components. The endogenous secretions are acid, pepsin and bile [8] which induce gastric damage. When the mucosal barrier is broken by the above materials, then gastric mucosa allows a back diffusion of gastric acid into the mucosal cells, leading to the mucosal damage [9] (Figure 1).

Acid constitutes the most important of the endogenous aggressive factors affecting the stomach lumen and a diminution in its production reduces most varieties of gastric mucosal injury [10]. In contrast, in the other side, acid can in some senses be viewed as the first line of mucosal defense, because it is important for reducing the possibility of bacterial colonization of the stomach and therefore the entry of bacteria into the systemic circulation when there is a breach in the gastric epithelium [8]. Furthermore, the first etiologic agent in stomach to cause ulcer includes hypoxia, which in most cases is due to a low blood flow or ischemia [11]. The lack of oxygen causes cell injury [12].

Most of the mechanism of pathogenesis of peptic ulcer has been elucidated, which has helped to the better understanding of gastroprotection and the searching for ulcer prevention and healing therapeutic drugs for peptic ulcer.

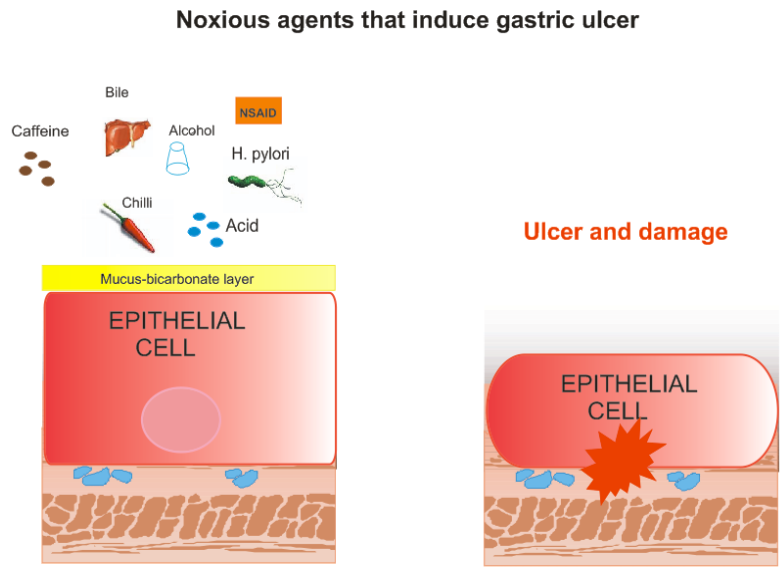


Figure 1. Noxious agents that stimulate peptic ulcer appearance

3. Gastroprotection

Gastroprotection is a term attributed to the ability of the gastric tissue to prevent injury. The prevention of this damage mainly consists on functional, humoral and neuronal factors. Mucus-alkaline secretions, the phospholipids layer, microcirculation and motility act as functional factors. Prostaglandins (PGs), nitric oxide (NO), lipoxins (LXs) and hydrogen sulfide (H_2S) work as humoral factors and capsaicin sensitive sensory neurons act as neuronal factor [4, 9, 13, 14]. Besides, a lack on leukotrienes (LTs), leukocyte adherence and tumoral necrosis factor (TNF-α) has been considered such as a gastroprotective mechanism [5, 15] (Figure 2).

3.1. Functional Factors

3.1.1. Mucus-bicarbonate-phospholipid barrier

Mucus and bicarbonate are secreted in the gastric epithelium [16]. The first line of mucosal defense is constituted by the mucus-bicarbonate-phospholipid "barrier". This barrier is formed by mucus gel, bicarbonate, and surfactant phospholipids, which cover the mucosal surface [17]. The gastrointestinal epithelial barrier helps to preserve mucosal integrity by preventing the entry of foreign particles of pathogens. This layer delays acid permeation into gastric epithelium, then bicarbonate can neutralize luminal acid, being that the first line of mucosal defense. Moreover, *Helicobacter pylori* is able to impaired mucus layer and consequently decrease pH on the epithelium [18]. Exogenous irritants exhibit the ability to diminish pH and decrease mucus secretion, and then induce injury by this mechanism.

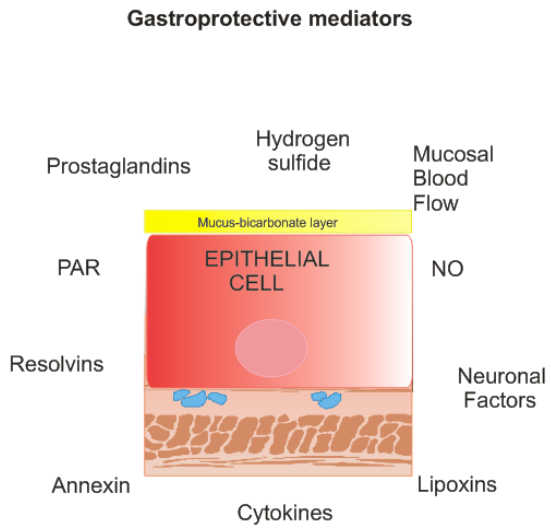


Figure 2. Gastroprotective mediators are divided into functional factors (mucus-alkaline secretions, the phospholipids layer, microcirculation and motility), humoral factors (prostaglandins (PGs), nitric oxide (NO), lipoxins (LXs) and hydrogen sulfide (H_2S)) and neuronal factors (capsaicin sensitive sensory neurons). While there are another factors such as diverse cytokines, proteinase activated receptor (PAR), annexin and probably resolvins that could act as protective mediators.

Mucus gel is secreted by apical expulsion from surface epithelial cells [19, 20]. The mucus bicarbonate barrier is the only preepithelial barrier between lumen and epithelium. The mucus gel is formed by phospholipids, and its luminal surface is coated by a film of surfactant phospholipids with strong hydrophobic properties [19]. When the mucosal barrier is overwhelmed or breaks down by injury, there is another mechanism that could be performed in the mucosal defense.

3.1.2. Mucosal microcirculation

Mucosal microcirculation is essential for delivery of oxygen and nutrients and removal of toxic substances. At the level of the muscularis mucosae, most gastric arteries branch into capillaries which enter to lamina propia and travel upward in proximity to gastric glandular epithelial cells. At the base of surface epithelial cells, capillaries converge into collecting venules [21].

When acid or other irritants enter the subepithelial compartment, sensory afferent neurons are able to trigger a rapid increase in mucosal blood flow that allows the buffering of acid and the rapid removal of toxic substances, thus limiting their penetration into deeper layers of the mucosa [22]. Mucosal blood flow is mediated by endogenous substances such as PGs, NO, H_2S and sensory afferent nerves [23], the role of those substances will be described in
section 3.2.

It has been described that sildenafil, a cyclic GMP-specific phosphodiesterase inhibitor promotes an increase in $_c$GMP concentrations in the gastrointestinal tract; besides is well known that $_c$GMP mediates many of the biological actions of NO, such as promoting an increment on gastric blood flow. Sildenafil increases mucosal defense against indomethacin-

induced gastropathy in rats, and this effect is reversed by concomitant administration of *L*-NAME (an inhibitor of NO synthesis). This mechanism is mediated by the reduction of leukocyte adhesion and maintenance of gastric blood flow [24].

Functional maintenance of gastric blood flow plays an important role in gastric mucosa defense [25]. This maintenance of normal gastric blood flow occurs even in the presence of damaging agents such as NSAIDs or ethanol [24, 25].

3.1.3. Motility

Normal gastric motility has been related with the mucosal gastric defense. NSAIDs induce gastric injury throw PGs inhibition. Those drugs induce hipermotility which is an important step for gastric damage induction. Furthermore, gastric hipermotility induced by NSAIDs is associated with a PG deficiency caused by COX-1 inhibition; this was demonstrated in some experiments where gastric hipermotility was induced after indomethacin and SC-560 (COX-1 inhibitor) administration but not with rofecoxib (COX-2 inhibitor) treatment [26]. Moreover, sildenafil modifies gastroduodenal motility in both humans [27] and animals [28].

The basis for the protection from gastric motility is a decrement on it. Glucorticoids are assumed to protect gastric mucosa via their maintenance of glucose homeostasis, gastric blood flow, and mucus secretion and their attenuation of enhanced gastric motility and microvascular permeability [29]. It is thought that gastric hipermotility event decreases gastric blood flow, explaining its pathogenicity.

3.2. Humoral Factors

3.2.1. Prostaglandins (PGs)

The first knowledge of prostaglandins occurred in 1930 with the observations of von Euler and Goldblatt of some substances in semen that cause smooth muscle contractions; their name come due to these substances where study by first time in prostate and was proposed during Bergström, Samuelson and co-workers studies, who determined prostaglandins structures. During 1971, Sir John Vane and colleagues discover the action of aspirin and related anti-inflammatory drug as inhibitors of PGs.

Prostaglandins are eicosanoids derived from arachidonic acid by the initial action of cyclooxygenase (PG endoperoxide G/H synthase). Arachidonic acid is a fatty acid derived from dietary sources or is synthesized in the body from an essential fatty acid, linoleic acid. It is stored in lipid bilayers of cell membranes and is esterified predominantly to phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol by an enzyme named phospholipase A_2 (PLA_2) [30].

Prostaglandins are divided into series that differ in the oxygen substitution in the cyclopentane ring and coded by a letter (PGD, PGE, PGF, PGG, and PGH). The subscript numeral in PG nomenclature indicates the number of double bonds present in the compound [30]. Cyclooxygenase (COX) is a heme-containing enzyme that is most abundant in the endoplasmic reticulum. There are two major isoenzymes COX-1 and COX-2 and catalyzes two reactions: cyclization of arachidonic acid to form PGG_2 and hydroperoxidation of PGG_2

to yield PGH_2 [31]. The latter is a relatively unstable compound that has a half-life of seconds and is a common intermediate that is converted to biologically active products such as thromboxane (TXA_2), prostacyclin (PGI_2), PGD_2, PGE_2 and $PGF_{1\alpha}$, by thromboxane synthase, prostacyclin synthase, PGD_2 isomerase, PGE_2 isomerase and PGF reductase respectively. The synthesis of each kind of prostaglandin depends of the cell and enzyme present in each tissue.

In gastric tissue, mainly are synthesized PGE_2 and PGI_2. Prostaglandins derived from COX-1 in gastric mucosa mediate many of the components of gastric mucosa defense, such as the maintenance of gastric blood flow by PGI_2 and bicarbonate and mucus secretion by PGE_2; in addition it retards the ability of acid and pepsin to penetrate mucus [22, 32]. When COX-1 is inhibited, COX-2 expression is induced to protect or heal gastric damage. COX-2 synthesizes PGs from arachidonic acid and their functions in gastric mucosa healing are due to the inhibition of leukocyte adherence and the increment on epithelial proliferation [32]. Prostaglandins generally act in an autocrine or paracrine manner and have short half-lives (seconds to minutes) in the circulation.

COX-1 is the predominant form expressed in the normal gastrointestinal tract, but COX-2 can be detected and has been shown to be rapidly up-regulated in response to a number of stimuli, such as aspirin or indomethacin administration [33].

Besides, COX-1 and COX-2 inhibition is required for the development of gastric erosions after NSAID administration [34, 35]. It has been reported that SC-560, a COX-1 selective inhibitor, did not elicit gastric damage, even SC-560 decreased gastric blood flow and did not increase leukocyte adherence in mesentery. Furthermore, celecoxib, a selective COX-2 inhibitor, did not induce gastric damage by itself. However, celecoxib increase leukocyte adherence, while did not produce any significant changes in gastric blood flow. Celecoxib and SC-560 administered concomitantly induced a decreased on gastric blood flow and an augmented leukocyte adherence, and then this combination produced gastric erosions [35].

Moreover, mice in which the gene for COX-1 was disrupted did not exhibit spontaneous gastric damage despite negligible gastric PG synthesis. However, these mice did developed erosions when indomethacin was given (a dual COX-1/COX-2 inhibitor) [34].

Furthermore about mucosal defense role of COX-2, this enzyme derived prostaglandins also make an important contribution to the repair of ulcers. COX-2 is strongly expressed in cells at the ulcer margin, which is where epithelial proliferation primarily occurs, allowing for reestablishment of glands. COX-2 is also strongly expressed in endothelial cells in the ulcer bed, where is the site of new vessel growth (angiogenesis) [36].

3.2.2. Nitric oxide (NO)

Nitric oxide is a small molecule synthesized from the terminal guanidine nitrogen atom of L-arginine. Its synthesis is carried out by a nitric oxide synthase (NOS) through a five electron oxidation reaction using as cofactors flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin and protoporphyrin IX heme. There are three different isoforms of NOS, its distributions depends on cell kind. Two of them are Ca^{2+}/calmodulin-dependent constitutive enzymes ($_c$NOS); neuronal ($_n$NOS) and endothelial ($_e$NOS). The third

enzyme is inducible ($_{i}$NOS), which is Ca^{2+}-independent and is induced by exposure to cytokines and lipopolysacharide in various cells types such as inflammatory cells [10].

Since the discovery of the vasodilator properties of NO by Nobel in nitroglycerine, NO has been implicated in several studies about its relaxing properties in endothelium. In particular in gastric mucosa NO interacts with neuropeptides and prostaglandins to maintain mucosal integrity in basal conditions. However, inhibition of NO synthesis alone does not cause gastric damage; lesions appear if this treatment is combined with ablation of sensory neurons following treatment with capsaicin or with non-ulcerogenic doses of indomethacin [10]. This gas mediator participates in gastric mucosa defense by regulating gastric mucosa blood flow, acid and alkaline and mucus secretion [37].

Furthermore, it has been reported that a diet rich in nitrates increases gastric blood flow. Nitrate is absorbed in the proximal small intestine and then concentrated in the salivary glands [38]. Salivary nitrate is then reduced to nitrite by oral bacteria and is further reduced to NO in the acidic stomach [39, 40]. Besides, studies have demonstrated that application of a solution of NO or a NO donor to the mucosa protected from injury [41].

It has been described that NSAIDs induced gastric injury by the inhibition on the synthesis of gastric prostaglandins. Prostaglandins exhibit their gastric protective effect by the increment on gastric blood flow. Furthermore, NO induces the increment on gastric blood flow. Nitric oxide-releasing NSAIDs have been developed to release NO and this substance could compensate the lack on prostaglandins synthesis. For example, a nitric oxide-releasing derivative of naproxen, HCT-3012 [(*S*)-6-methoxy-α-methyl-2-naphtalene-acetic acid 4-(nitrooxy)butyl ester], similar to naproxen inhibits synthesis of prostaglandins derived of COX-1 and COX-2 [42]. However, HCT-3012 has been related with fewer lesions in gastric mucosa in healthy human volunteers than those volunteers administered with naproxen [43]. Other nitric oxide-releasing NSAIDs such as aspirin derivative and flurbiprofen derivate have not induced gastric damage even though they inhibit prostaglandin synthesis [44, 45].

Moreover, NO-releasing NSAIDs induces a lack on the increment on leukocyte adherence, while conventional NSAIDs increase leukocyte adherence in the mesentery. This is a compensatory effect for the inhibition in prostaglandins, and then NO-releasing aspirin derivative reduces the susceptibility of the stomach to shock-induced damage through inhibitory effects on neutrophil adherence to the vascular endothelium. [46].

3.2.3. Lipoxins

Lipoxins are trihydroxytetraene-containing lipid mediators; they result from the sequential oxygenation of arachidonic acid at the carbon-15 and the carbon-5 position by 15- and 5- lipooxygenase, respectively. Lipoxins are characterized structurally by the presence of four conjugated double bonds [30]. Lipoxins are formed during cell-cell interactions and are predominantly counter regulators of some mediators of inflammation [47].

Lipoxins are known to be generated in humans by one of at least three biosynthetic routes working independently or in concert, in particular biological settings or tissues. The first route occurs between airway epithelial cells or monocytes with neutrophils. The second pathway is induced by interactions predominantly within the vasculature between 5-LO, present in myeloid cells, and 12-LO, present in platelets.

The third route involves aspirin and the action of cyclooxygenase (COX-2) and 5-LO (lipooxygenase) [48]. Endothelial and epithelial cells express COX-2 in response to various stimuli such as cytokines, hypoxia and bacterial infections [47]. Aspirin covalently modifies, through acetylation, a serine residue near the active site of COX. In the case of COX-1, this acetylation occurs in a serine residue near the active site (Ser530) which induces a conformational change in the enzyme, and then it can no longer oxidize arachidonic acid [49]. With COX-2 does not occur the same, aspirin also acetylates a serine residue (Ser516), but COX-2 remains able to metabolize arachidonic acid to 15-(*R*)-HETE (15-*R*-hydroxyepitetraenoic acid) [50]; this compound is released from endothelial and epithelial cells and transformed by leukocyte 5-LO to 15-epimer lipoxin A_4 or aspirin-triggered lipoxins (ATL) [47]. Those events could occur in endothelial cells from mesentery and vessels in gastric microcirculation; furthermore, lipoxins exert potent protective actions on the gastric mucosa. Lipoxin generation in gastric tissue after aspirin administration was induced throw COX-2 activity; concomitant administration with a COX-2 inhibitor exhibits greater gastric damage after aspirin administration. These effects may occur in part through the ability of lipoxins to suppress aspirin-induced leukocyte adherence within the gastric microcirculation [14]. Lipoxins produce their effects via the FPRL-1 receptor [51], blockade of this receptor results in a significant increment of the gastric damage effects of aspirin [14].

Besides, lipoxins inhibit LTB_4 responses in neutrophils by down-regulating CD11b/CD18, and then reduce leukocyte adhesion to the endothelium an early step for gastric damage pathogenesis [52]. Moreover, nitric oxide exhibits its anti-inflammatory effects in the microcirculation by inhibiting leukocyte-endothelium interactions, and lipoxins increases nitric oxide synthesis through $_{e}$NOS and $_{i}$NOS [53]. In addition, Wallace and coworkers evaluated the effect of intraperitoneal administration of synthetic LXA_4 prior to oral administration of aspirin to determine if ATL might act to reduce the severity of aspirin-induced gastric mucosal injury. They found that LXA_4 dose-dependently reduced aspirin-induced injury in the stomach [14]. The most important event than lipoxins offer to gastric safety are the increment on mucosal blood flow and the decrement on leukocyte adherence to the vascular endothelium, an early step on pathogenesis in gastric mucosa induced by NSAIDs [54].

3.2.4. Resolvins

The essential polyunsaturated fatty acids (PUFA) include arachidonic acid of the ω-6 series, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from the ω-3 series of PUFA [55].

Similar as occurs with lipoxins, aspirin acetylates Ser516 in the internal cavity of COX-2 active site to cause a shift in the position and chirality of oxygen insertion by a change in the conformation of the omega side chain [56]. It has been reported that aspirin treatment of COX-2 enhanced the production of 15-*R*-HETE from araquidonic acid to form lipoxins, 18-*R*-HEPE from EPA and 17-*R*-HDHA from DHA. When 18-*R*-HEPE and 15-*R*-HEPE were incubated with activated human PMN were converted to trihydroxy-containing EPE compounds, namely 5*S*,12*R*,18*R*-triHEPE (Resolvin E1, RVE1) and 5*S*,6*R*,15*R*-triHEPE (15-epi-lipoxin A_5).

RvE1 owns counter regulatory actions to inhibit PMN transendothelial migration *in vitro* and also acts as a potent inhibitor of leukocyte infiltration [57]. Furthermore, administration of EPA and aspirin in a model of mouse peritonitis model induced RvE1 generation in exudates and reduced leukocyte infiltration. Also, in a model of colitis, RvE1 protected against the damage by the decrement of leukocyte infiltration and proinflammatory gene expression of TNF-α and other cytokines [58].

Resolvins has not been studied as a gastroprotective substance, however considering that an increment on leukocyte adherence is involved in the pathogenesis for gastric injury for several compounds and resolving anti-inflammatory properties. Resolvins could exert a protective effect on compounds-induced gastric injury. This statement should be explored.

3.2.5. Hydrogen sulfide (H_2S)

Hydrogen sulfide is synthesized endogenously from L-cysteine primarily via two enzymes: cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS). In some tissues, CSE and CBS are both required for H_2S synthesis, whereas in others only one enzyme is necessary [59]. CBS and CSE are expressed to different extents in neurons in brain and in the enteric nervous system in the gut. H_2S was studied first in vascular smooth muscle, where as NO, H_2S exhibits relaxing effect by direct action on ATP-sensitive K^+ channels [60]. CSE and CBS are expressed in gastric mucosa and endogenous H_2S apparently plays the role of a protective factor against mucosal injury; H_2S regulates gastric mucosal blood flow and leukocyte adherence to the vascular endothelium [4]. Furthermore, aspirin and other NSAIDs reduce H_2S generation by directly modulating the expression activity of CSE and a releasing of H_2S (NaHS) protects against the reduction of mucosal blood flow cause by aspirin. Glibenclamide, a K_{ATP} blocker reduced the anti-adhesive effects of H_2S, whereas pinacidil, a K_{ATP} opener, protects against mucosal injury caused by aspirin [4]. K_{ATP} channels mediate gastric mucosa homeostasis [61].

Anti-inflammatory drugs have been synthesized to release H_2S, for example a derivate of diclofenac (ATB-337), which is linked to a H_2S-releasing moiety, spares gastrointestinal mucosa of injury. This compound did not stimulate leukocyte adherence to the vascular endothelium of postcapillary mesenteric venules, in contrast to the effects of diclofenac [5], this event is related with a lack on the increment in gastric granulocyte infiltration or expression of leukocyte or endothelial adhesion molecules.

Furthermore, TNF-α contributes to gastric injury induced by NSAIDs and alcohol. ATB-429, reduces the expression of many proinflammatory cytokines, while it did not change IL-10 expression, an anti-inflammatory cytokine. ATB-429 consists of a molecule of mesalamine linked via an ester bond to a molecule of ADT-OH [62]. ADT-OH has been shown to liberate H_2S when incubated in buffer and even greater generation was observed when it was incubated on homogenate liver [63].

Besides, NaHS, a donor of H_2S, possesses a dual effect on H_2O_2-caused cell death in mucosal epithelial cells. This was performed in the experiments where NaHS induced a strong protective action at 1.5 mM but slight aggravation of the toxicity at 0.5-1 mM. With those results, it can be concluded that NaHS may directly protect gastric mucosal epithelial cells against oxidative stress, and further studies give the tools for the elucidation that this via

is throw activation of the ERK (extracellular signal-regulated kinase) and JKN (Jun N-terminal kinase) pathways [64].

Additionally H_2S role of protecting gastric mucosa from injury, it has been related with ulcer healing. Twice-daily treatment for a week with hydrogen sulfide donors increased extent of healing gastric ulcers after acetic acid-induced gastric injury. *L*-cysteine, a precursor of H_2S, also accelerates healing of the ulcers. Taking together, these results suggest that hydrogen sulfide is produced in the gastric mucosa response to injury and acts to promote healing [13]. Ulcer healing may be related with the increment on VEGF (vascular endothelial growth factor) [65].

Even though there are several reports about the anti-inflammatory and gastroprotective effect of hydrogen sulfide, it has exhibited a paradoxical role. Hydrogen sulfide participates as proinflammatory substance; sodium hydrosulfide (donor of H_2S) in mice increased lung and liver mieloperoxidase activity and raised TNF-α concentration, while *D-L*-propargylglycine (a CSE inhibitor) exhibited marked anti-inflammatory activity [66]. H_2S-producing enzymes are presented on enteric neurons of humans and guinea pig; H_2S evokes ion secretion in colonic mucosa by activating TRPV1 (transient receptor potential vanilloid-1) on extrinsic primary afferent terminals [67].

Further studies needs to be done to elucidate the mechanism for the dual role of hydrogen sulfide acting as pro-inflammatory and anti-inflammatory molecule.

3.3. Neuronal Factors

The enteric nervous system is a collection of neurons in the gastrointestinal tract that constitutes the "brain of the gut" and can function independently of the central nervous system. This system controls the motility, exocrine and endocrine secretions, and microcirculation of the gastrointestinal tract; it is also involved in regulating immune and inflammatory processes [68].

There are two principal intramural plexuses in the gastrointestinal tract: the *myenteric* plexus (Auerbach's plexus) and the *submucosal* plexus (Meissner's plexus) on the luminal side of the circular muscle layer. Preganglionic and parasympatic fibers from the vagus are connected to ganglion cells in the plexuses.

The neurons within the plexuses constitute the *enteric nervous system* and secrete acetylcholine, noradrenaline (norepinephrine), 5-hydroxytryptamine, purines, nitric oxide and a variety of pharmacologically active peptides such as gastrin-releasing peptide (GRP), vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP) and substance P. The enteric plexus also contains sensory neurons, which respond to mechanical and chemical stimuli [3, 69].

When mucosal barrier is disrupted an increment of acid in the lamina propia is the signal key to spinal afferent neurons activates a peptide transmitter, calcitonin gen receptor peptide (CGRP) to induce NO synthesis, finally NO increases gastric blood flow and bicarbonate secretion [70].

Furthermore, CGRP in nerve fibers has been related with the capacity of the gastric mucosa to defend itself against injury [71]. TRPV1 is an acid-sensitive ion channel expressed

by vagal and spinal afferent neurons innervating the rodent and human GI tract [72, 73]. Capsaicin, the main active ingredient on hot chilli peppers, activates TRPV1 [74]. In humans, capsaicin decreased stomach injury caused by ethanol and microbleeding induced by indomethacin administration was reversed by co-administration with capsaicin [75].

Capsaicin induces its gastroprotective action by the induction of the increment on gastric blood flow, but in contrast to this statement capsaicin reverts gastroprotection induced by tumoral growth factor-α (TGF-α) [76]. Then, capsaicin exerts its gastroprotective effect at low doses, while higher doses of capsaicin induce injury. Taken those results together, appear that capsaicin is involved in adaptative cytoprotection induced by mild irritants. Adaptative cytoprotection is the ability that gastric mucosa possesses to induce damage by prolonged exposure to low doses of an irritant.

3.4. Other Gastroprotective Mechanism.

3.4.1. Cytokines

Cytokines are substances released from the immune system to cause injury or healing. IL-1β is produced in various types of cells such as monocytes, macrophages, neutrophils, endothelial cells and fibroblasts [77]. IL-1β inhibits migration of neutrophils and leukotriene B_4 in a dose-dependently manner after the injection of the noxious stimulus. IL-1β also protected the mucosa against indomethacin-induced injury in gastric tissue [78]. This interleukin also reduced acid gastric secretion induced by aspirin treatment [79] and inhibited the release of platelet-activating factor, a potent pro-inflammatory substance, from peritoneal mast cells through stimulating NO release [80].

3.4.2. Annexin-1

Annexin-1 is a protein of 37 kDa, which used to be named as lipopocortin-1. Annexin-1 is member of annexin family of proteins that bind to and activate “formyl-peptide” receptors (FPR) [81, 82]. Glucocorticoids can modulate this protein expression. It was found that annexin-1 possesses calcium and phospholipids binding properties and was actively involved in the inhibition of eicosanoid synthesis and PLA_2 [83].

Furthermore, glucocorticoid treatment increases annexin-1 content in circulating neutrophils in humans and rodents [84, 85]. Annexin-1 promotes leukocyte detachment, and then inhibits cell extravasation. Besides, dexamethasone, a glucocorticoid, protects the mucosa against indomethacin-induced injury. This effect was reverted by the administration of formyl-peptide receptor antagonist. Dexamethasone decreases leukocyte adherence in mesentery induced after indomethacin treatment, throw the expression of annexin-1; besides, annexin-1 is expressed constitutively in rat stomach [86]. Annexin-1 has been related to act throw the same receptor than lipoxin A_4 and 15-epi-LXA_4.

Annexin-1 also participates on gastric ulcer healing; annexin-1 expression is strongly induced in ulcerated gastric tissue. Furthermore, annexin-1 knockout mice show similar susceptibility to indomethacin-induced gastric damage. For this result Martin proposes the hypothesis that annexin-1 contributes to the healing of gastric mucosal damage [83].

3.4.3. Proteinase-activated receptors (PAR)

There are four proteinases elucidated from today. PAR-1, PAR-3 and PAR-4 are activated by thrombin, while PAR-2 is activated by trypsin or human mast cell tryptase. PAR-2 is expressed in the gastrointestinal tract, including on epithelial cells and sensory afferent neurons [87]. PARs are G protein-coupled receptor; those proteins are activated by proteolytic unmasking of the N-terminal extracellular tethered ligand that presumably binds to the extracellular loop 2 of the receptor itself [88]. PAR-2 agonist triggers mucus secretion on stomach but not in duodenum and prevents gastric injury originate by HCl-ethanol or indomethacin. PAR-2 triggers its cytoprotective secretion of gastric mucus by stimulating the release of CGRP, due to capsaicin secretion abolished mucus secretion induced by PAR-2 [88].

Activation of PAR-1 releases VEGF (vascular endothelial growth factor) from platelets, which promotes new blood vessels growth (angiogenesis); furthermore PAR-1 inhibits release of endostatin an inhibitor of the growing of new blood vessels. The growth of new blood vessels in the margin of ulcer helps to heal it [89].

3.3.4. LTB_4 and leukocyte adherence

Leukocyte adherence contributes to the pathogenesis of gastric mucosal injury in two ways. First, leukotriene B_4 leads the increment on leukocyte adherence and at the same time permits the liberation of oxygen-derived free radicals and proteases. Second, neutrophil adherence to the vascular endothelium could obstruct capillaries, resulting in a reduction in gastric mucosal blood flow and thereby predisposing the mucosa to injury [90]. Asako studied the role of LTB_4 in leukocyte adherence after indomethacin administration. In this study, indomethacin induced the increment on leukocyte adherence; leukocyte adherence was abolished after pretreatment with a LTB_4 antagonist. Taking those results together suggest that indomethacin induced gastric injury throw the increment on leukocyte adherence, a dependent mechanism from the increment on LTB_4 [91]. Furthermore, this appeared to be mediated for the increment in the expression of intercellular adhesion molecule 1 (ICAM-1) due to pretreatment with an antibody against ICAM-1 reduced leukocyte adherence and susceptibility to NSAID-induce gastric damage [92].

Lately, the gastroprotective effect of many substances has been related with the decrement on LTB_4 production and leukocyte adherence at basal levels. NO, H_2S, PGs-derived from COX-2 maintain basal leukocyte adherence levels. The attachment of NO and H_2S to the molecule of an NSAID, commonly named such as nitric-oxide releasing NSAIDs and hydrogen sulfide-NSAID contributes to the decrement on gastric injury-induced by NSAID. This diminish in gastric injury is by the decrement on LTB_4 production and leukocyte adherence [5, 24, 43]. Besides, lipoxins exerts their gastroprotective effect by the reduction on leukocyte adherence in mesentery [14].

However, not all traditional NSAIDs cause gastric injury; recently, acemetacin, a prodrug of indomethacin demonstrated to induce gastric safety against to its biotransformation to indomethacin. Acemetacin induced inhibition of PGs as traditional NSAIDs but it did not induce the increment on LTB_4 or leukocyte adherence, then its gastric safety is related with the lack on the increment on LTB_4 and leukocyte adherence [15].

Gastroprotective Mechanism

Mediator	Response
Functional factors	
Mucus-bicarbonate-phospholipid "barrier"	Preserve mucosal integrity by preventing the entry of foreign particles of pathogens
Mucosal microcirculation	Delivery of oxygen and nutrients and removal of toxic substances from the mucosa
Humoral factors Prostaglandins (PGs)	Maintenance of gastric blood flow by PGI_2 and bicarbonate and mucus secretion by PGE_2 Inhibition of leukocyte adherence and the increment on epithelial proliferation by PGs-derived from COX-2
Nitric oxide (NO)	Regulation of gastric mucosa blood flow, acid, alkaline and mucus secretion. Inhibition of leukocyte adherence
Lipoxins	Suppress aspirin-induced leukocyte adherence within the gastric microcirculation. Inhibiton of LTB_4 responses in neutrophils Down-regulates CD11b/CD18, and reduction of leukocyte adhesion to the endothelium Increment of nitric oxide synthesis Increment of mucosal blood flow
Hydrogen sulfide (H_2S)	Inhibition of leukocyte adherence Reduction in the expression of proinflammatories cytokines such as TNF-α Increment of mucosal blood flow
Neuronal factors Calcitonin gen receptor peptide (CGRP)	Induction of NO synthesis and increment on gastric blood flow
Other gastroprotective factors Cytokines	IL-1β inhibits migration of neutrophils and leukotriene B_4 Inhibition in the release of platelet-activating factor
Annexin-1	Promotion of leukocyte detachment, and inhibition of cell extravasation Role on gastric ulcer healing Activation of the same receptor than lipoxin A_4
Proteinase-activated receptors (PAR)	PAR-2 triggers cytoprotective secretion of gastric mucus by stimulating the release of CGRP PAR-1 releases VEGF and inhibits release of endostatin

3.3.5. TNF-α

Tumour necrosis factor α is expressed after NSAID and ethanol administration, and it has been related with their adverse effects [4, 7, 15]. This event is a stimulus for the expression of

adhesion molecules. Pretreatment with pentoxifylline, an inhibitor of TNF-α synthesis, dose-dependently reduced neutrophil accumulation in the gastric microcirculation and gastric damage [93]. Moreover, acemetacin treatment also decreases TNF-α production and it is related with its gastric safety [15]. The development of new compounds without the induction on the expression on TNF- α could help to enhance the gastroprotective properties of some drugs.

There are many factors that can play the role as gastroprotective substances. The most studied of them are NO, PGs, and recently lipoxins and H_2S. These substances regulate in orchestra the gastric mucosal inflammation. When the absence of one of them is induced, the over-expression of the other ones is produced to compose this decrement and maintain the mucosal gastric defense.

4. Experimental Models to Study Gastroprotection

An experimental model for the study of gastroprotection requires the induction of mucosa injury with the less as possible suffering of the animal. Between all the experimental models the induction of injury for ethanol absolute and NSAIDs administration are the most popular. However, administration of acidified ethanol (HCl:EtOH), NaOH, stress-induced ulcer, pylorus ligation and acetic acid are used depending of the mechanism and expected results from the researcher. In this chapter, we will describe the gastric damage induce by ethanol and NSAIDs administration and the parameters that could be measured.

4.1. Ethanol Induce Gastric Damage

Oral administration of ethanol has been described to induce gastric damage; the severity of the injury is related with the doses of ethanol. Absolute ethanol induces severe histopathological changes in oxyntic mucosa of mouse and rat stomach consisting of acute erosive hemorrhagic lesions, vascular congestion, edema and necrosis [94, 95]. Furthermore, ethanol causes depletion of the gastric levels of proteins, nucleic acids, NP-SH (non-protein sulfhydryl groups) and an increment on MDA (malondialdehyde) levels and decrement of antioxidants substances [96].

Moreover, it has been described that depletion of NP-SH groups by ethanol increases the content of free radicals mediate tissue injury by stimulating lipid peroxidation and membrane damage [94].

Low doses of ethanol can induce damage as well; for example, administration of 25 % of ethanol induced a decrement on mucus secretion and an increment on the acid juice secretion [97]. However, this effect has been related lately with the termed cytoprotection adaptative. Furthermore, administration of 50 % of ethanol induces injury by constriction of venules and this effect is reverted by prostaglandin exogenous administration [98]. Absence of blood flow develops extensive gastric mucosa damage within a short period of time after contact with absolute ethanol. In contrast, no changes in blood flow exhibits no injury after ethanol administration [99, 100].

Those results together suggests that blood flow plays an important role in the pathogenesis of ethanol-induced gastric injury; studies by intravital microscopy have shown that damage occurs first by submucosal venular constriction, followed by cessation of mucosal blood flow and later mucosa necrosis [101]. How it has been seen with NSAIDs, on ethanol-induced gastric injury the decrement on gastric blood flow appears after an increment on leukotrienes [102] which may obstruct mechanically blood flow and recruits leukocytes that make worst the damage. Besides, mieloperoxidase activity (MPO, a marker of neutrophil infiltration) increased after ethanol administration. MPO increment correlates with ethanol-induced gastric injury [103].

TNF-α has been related in ethanol-induced gastric mucosa injury due to it cause inflammation and its synthesis is inhibited by cytoprotective prostaglandins. Furthermore, inhibition of TNF- α decrease ethanol-induced gastric injury [7] and pentoxyfilline a TNF- α inhibit neutrophil migration, being this a step to produce gastric damage [104]. In addition, exposure of the gastric mucosa to 40% of ethanol caused an increment in plasma TNF- α levels [7].

In summary, the damage induced by ethanol destroys the mucosa and submucosa caused by necrosis. The low levels of prostaglandins observed after ethanol administration is not due to cyclooxygenase inhibition, this should be more related with the necrosis of epithelial cells by direct contact with ethanol; epithelial cells are the responsible for prostaglandin secretion.

4.2. NSAIDs-Induced Gastric Damage

NSAIDs induced gastric damage due to their ability to inhibit prostaglandin synthesis, and it has been established in various studies [105, 106]. There are a correlation between time and dose dependent manner of suppression of gastric prostaglandin synthesis by NSAIDs and their ability to induce gastric ulcers [105].

The most common alterations caused by NSAIDs in gastric area are hemorrhagic gastric erosions, found more often in the fundus and corpus. While gastric ulcers in the antrum, are of greater clinical importance than erosions, due to their chronicity and the potential for perforation and bleeding [107].

As it has been described previously COX-1 is the predominant form expressed in the normal gastrointestinal tract [109]; however, COX-2 is rapidly up-regulated in response to a number of stimuli, such as administration of aspirin of indomethacin or following a period of ischemia [108, 109]. COX-1 participates in the secretion of mucus and bicarbonate and in the increment on gastric blood flow, while COX-2 is enrolled in the decrement of leukocyte adherence and re-epithelization of gastric cells [32]. Furthermore, COX-2 plays a very important role in ulcer healing [36]. Then, inhibition of COX-1 by NSAIDs reduces gastric mucosal blood flow [110]. Prostaglandins are potent vasodilators that are continuously produced by the vascular endothelium. NSAIDs also produce damage to the vascular endothelium and this is a very early step to induce injury in the gastrointestinal tract [33, 111] (Figure 3).

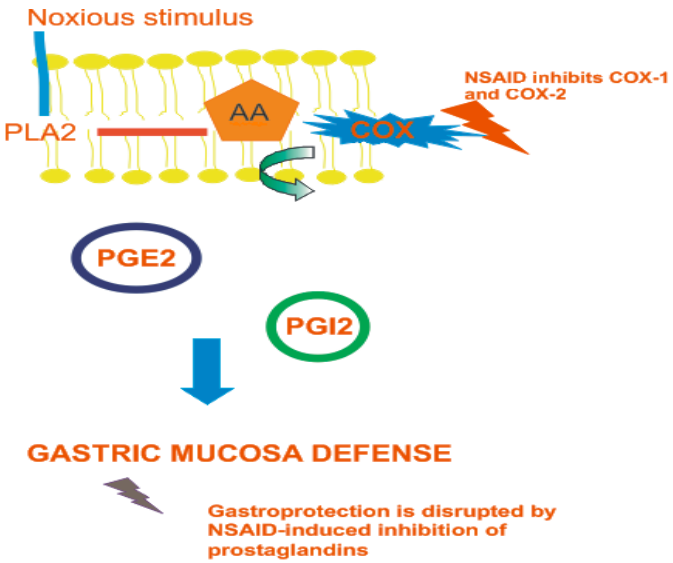


Figure 3. Phospholipase A_2 (PLA_2) synthesizes arachidonic acid (AA) from membrane phospholipids. Cyclooxygenases metabolizes AA to different prostaglandins in gastric mucosa PGE_2 and PGI_2 are bioconverted to increase mucosal gastric blood flow, increase mucus and bicarbonate secretion by COX-1. While prostaglandins synthesized from COX-2 participates decreasing leukocyte adherence and in re-epithelization of gastric cells. When an NSAID is administered, prostaglandins synthesis is inhibited by NSAID-inhibit COX and then gastroprotection is disrupted.

Besides, the increment on leukocyte adherence induce by NSAIDs is another important step in the pathogenicity caused in gastric mucosa. Treatment with monoclonal antibodies that blocked neutrophil adherence to the vascular endothelium markedly attenuated the severity of NSAID gastropathy in rats and rabbits [112, 113]. The augmented leukocyte adherence in mesentery is induced in an early step by leukotrienes due to inhibitors of leukotriene synthesis or antagonist of leukotriene receptors have been shown protective effects in experimental NSAID-induced gastric damage [112, 113]. Also, it has been studied the leukotriene B_4 levels after NSAID administration to rats [15] and humans [114]. Furthermore, inhibitors of leukotriene B_4 exert a decrement on leukocyte adherence to venules induce by NSAID [91].

Neutrophil adherence is regulated by endothelial expression of intracellular adhesion molecule 1 (ICAM-1) and selectin expression in neutrophil, those molecules allows the attachment of neutrophil to endothelial tissue [92, 115]. Another signal molecule that mediate NSAID-induced leukocyte adherence is the increment observed in TNF-α; TNF-α levels are increased in plasma after indomethacin administration to rats, and this correlates with the accumulation of neutrophils in the gastric microcirculation and the appearance of gastric injury [93].

Traditional NSAIDs inhibit COX-1 and COX-2, and then their gastric toxicity is induced by inhibition of prostaglandins derived from both enzymes. In the nineteen decade researchers thought that just COX-1 played the role of gastroprotective enzyme and COX-2 synthesized pro-inflammatory prostaglandins. With this premise coxibs (selective COX-2 inhibitors) were synthesized to reduce gastric injury induced by NSAID treatment [116].

However, some coxibs such as rofecoxib and valdecoxib have been withdrawn from the market due to their induced cardiovascular problems [32], such as heart broken. This event occurs because COX-2 inhibitors blocks prostacyclin (PGI_2) synthesis in endothelium, a vasodilator and anti-aggregative molecule; while COX-1 remains free to produce thromboxane (TXA_2) a vasoconstrictor and pro-aggregative substance. Furthermore, later was found that COX-2 plays a healing role in gastric mucosa, it is highly expressed in process where COX-1 is absent, and both isoforms of COX needs to be inhibited to cause gastric injury [35].

The solution at this moment for the abolishment of NSAIDs-induce gastric injury has been the attachment of NO or H_2S to the moiety of traditional NSAIDs such as naproxen, diclofenac and aspirin. Those molecules that release NO or H_2S have demonstrated to reduce the severity of NSAID-induce gastric damage throw the decrement on leukocyte adherence, adhesive molecules, TNF-α and the increment on gastric blood flow [5, 13, 24, 45, 63]. The studies in new molecules of NSAIDs donors of NO or H_2S could help to reduce NSAIDs gastric toxicity.

The main mechanism for NSAIDs to induce damage is the inhibition on the synthesis of prostaglandins. The absence of prostaglandins derive a decrement on mucus and bicarbonate secretion, increment on acid secretion, decreased gastric blood flow, an augment on leukocyte adherence and adhesive molecules. Those events are significant changes for the appearance of damage; the good management of them may help to diminish the pathogenecity of NSAIDs.

5. Gastroprotective Triterpenoids

Schmeda-Hirschmann and Yesilada in 2005 written a review of gastroprotective medicinal plants, however this review just focuses on crude drug or extracts [117]. Furthermore, another review was written by Borrelli and Izzo in 2000 [118]. Those review provide important and useful information, but at this moment after some years from the published work it has not been reported a review for gastroprotective triterpenoid and their mechanism. In this section we provide information of the work that has been developed in the latest years on the research of the gastroprotective properties and on the mechanism of gastroprotective action for some triterpenoids.

5.1. Triterpenoid Classification

Triterpenoids are a large group of natural products derived from C30 precursors. Triterpenoids with well-characterized biological activities include sterols, steroids and saponins. Ruzicka and co-workers deduced that all $C_{30}H_{50}O$ triterpene alcohols known were biosynthesized similarly, and then they proposed the biogenetic “isoprene rule” which explains the biosynthesis of all triterpene skeleton, the isoprene rule consist on the visibility of the skeleton of terpenes in isoprene units (molecule of 5 carbons) [119]. Furthermore, cyclization of squalene or oxysqualene has been the most credible origin of triterpenoids

[120]. The enzymes that catalyze these reactions are known as triterpene synthases and can be subdivided as squalene cyclases (SC) or oxysqualene cyclases (OSC), which convert squalene and oxyqualene to cyclic triterpenes and triterpene alcohols. Squalene was isolated by first time from the liver oil of shark (*Squalus* sp.). Then, it was found in rat liver and yeast.

Triterpenoids are classified according to number of cyclic ring in their chemical structure. Most triterpenoids are 6-6-6-5 tetracycles, 6-6-6-6-5 pentacycles or 6-6-6-6-6 pentacycles but acyclic, monocyclic, bicyclic, tricyclic and hexacyclic triterpenoids have also been isolated from natural sources.

Examples of monocyclic triterpenoid are achilleol A [121] and camelliol C [122]. Bicyclic triterpene have limited taxonomic distribution, species from Cratoxylum and Pistacia have bicyclic triterpene [123]. However, the experimental biosynthesis of these compounds has not been verified.

There is not evidence of natural tricyclic triterpenes, they just have been generated in the laboratory by *A. acidocaldarius* squalene-hopene cyclase mutant [124]. Lanosterol and cycloartenol are tetracyclic triterpenoid found in several plant sources. While lupine, germanicane, taraxastane, α-amyrin, β-amyrin and ursane are some examples of pentacyclic triterpenes [123].

5.2. Kind of Triterpenoids with Antiulcer Activity

Gastroprotective triterpenes have been isolated from several plants, for example lupeol acetate, ursolic acid, taraxerol were isolated from *Fabiana imbricata* [125, 126] and *Protium heptaphyllum* [127], and 18-β-glycyrrhetic acid from *Glycyrrhiza glaba* [128]. Which for clinical use 18-β-glycyrrhetic acid (enoxolon) was replaced by its soluble succinate sodium salt, carbenoxolone [129].

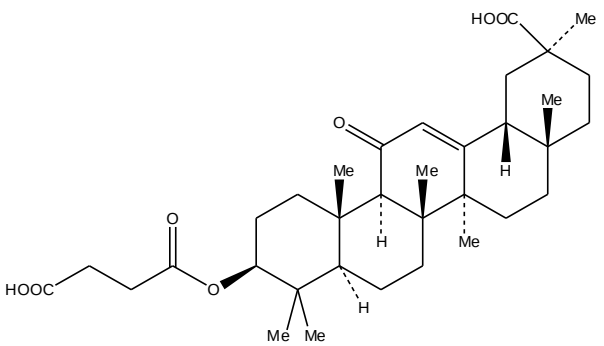
In regards to its chemical structure of triterpenoids, it has been proposed that a hydroxyl group at position C-3 (free or derivatised) is necessary for sterols and triterpenoids to exhibit antiulcer activity. This was proposed based in the experiments where the gastroprotective α-amyrin, β-amyrin, β-sitosterol and its glycoside, isolated from methanol extract of *H. excelsa* have a hydroxyl group at position C-3 on their chemical structure. In contrast, triterpenoids such as friedelin, cariophyllal and cariophillol do not possess the hydroxyl group in the position C-3 either they do not present gatroprotective activity [130].

Furthermore, oleanolic acid, ursolic acid, sericic acid and taraxerol are compounds that exhibit antiulcer activity and they contain a free hydroxyl group at C-3. Moreover, glycyrrhizic acid, carbenoxolone, lupeol acetate, sericoside and several triterpenoid saponins have a hydroxyl derivative at C-3 and protect gastric mucosa against injury [128].

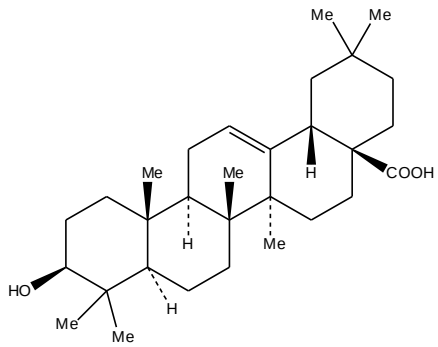
More triterpenoids have exhibited gastroprotective activity (Figure 4); for example, 3-*O*-acetyl aleuritolic acid has been reported as a constituent of several Euphorbiaceae species. This compound is the main component of *Croton cajuara* which reduces gastrointestinal transit in mice [131]. This substance was isolated from the rhizomes of the Paraguayan crude drug *Jatropha isabelli*, it exhibited gastroprotective activity in the HCl/EtOH induce gastric lesions in mice [132]. Besides, boswellic acid is an enriched mixture of tetra- and penta-

cyclic triterpenic acids isolated form the gum resin of *Boswellia serrata* with antiulcer activity [133].

Camellioside A and B, noroleanane-type triterpene oligoglycosides have produced a decrement on the lesions induce by ethanol and indomethacin administration [134]. Araloside A, a saponin triterpene is a potent inhibitor of gastric lesion in ulcer formation. This compound has been isolated from the root bark of *Aralia elata* [135].

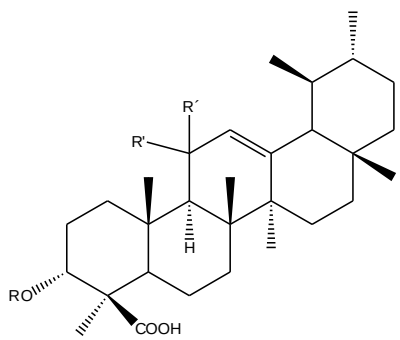


Carbenoxolone

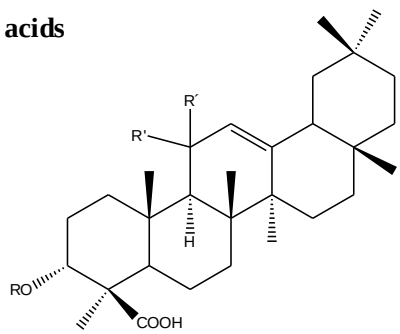


Oleanolic acid

Boswellic acids

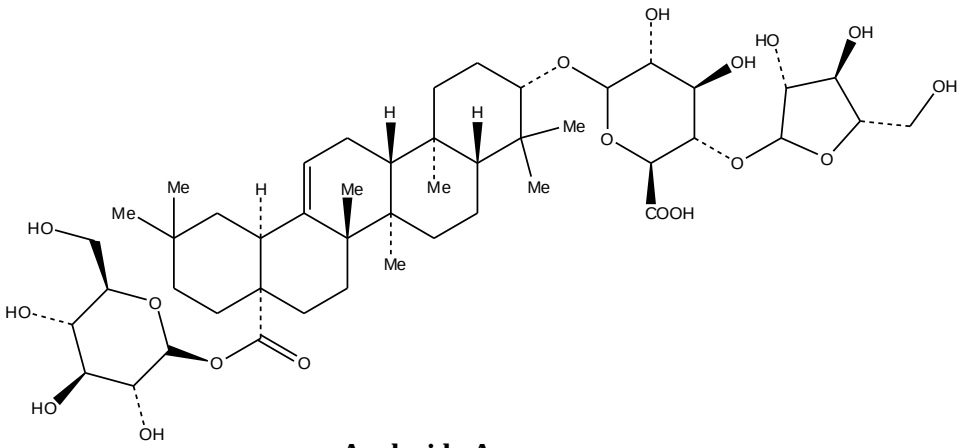


BA-1 R=H, R´= H_2 (β-Boswellic acid)
BA-2 R=OAc. R´=H_2 (Acetyl-β-boswellic acid)
BA-3 R=H R´=O (11-keto-β-boswellic acid)
BA-4 R=OAc. R´=O (Acetyl-11-keto-β-boswellic acid)

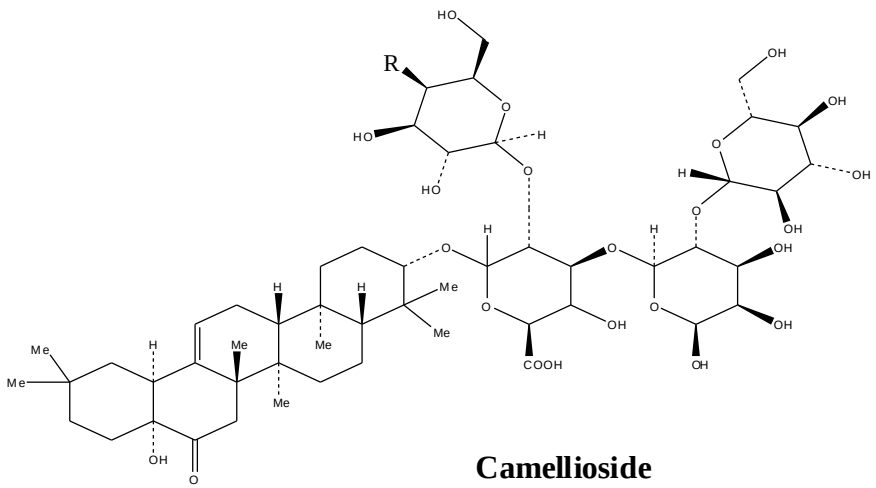


BA-1a R=H R´=H_2 (α-boswellic acid)
BA-2a R=OAc. R´=H_2 (Acetyl-α-boswellic acid)

Figure 4. Continued on next page.

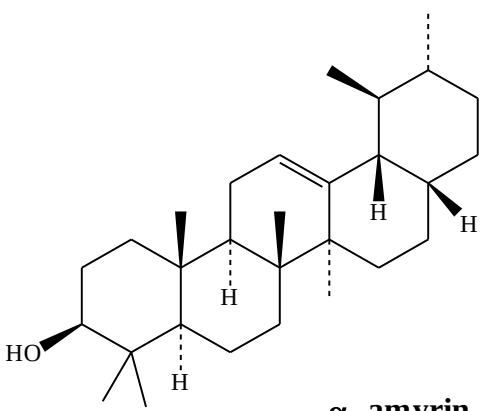


Araloside A

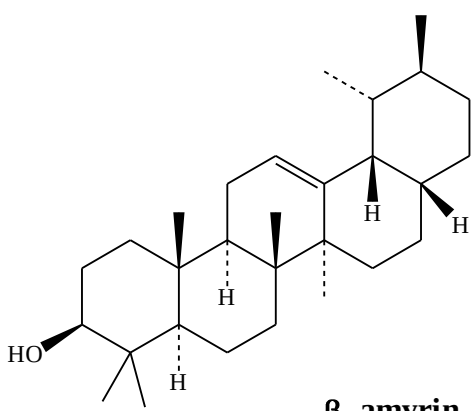


Camellioside

Camellioside A. R=OH
Camellioside B. R=OAc



α–amyrin



β–amyrin

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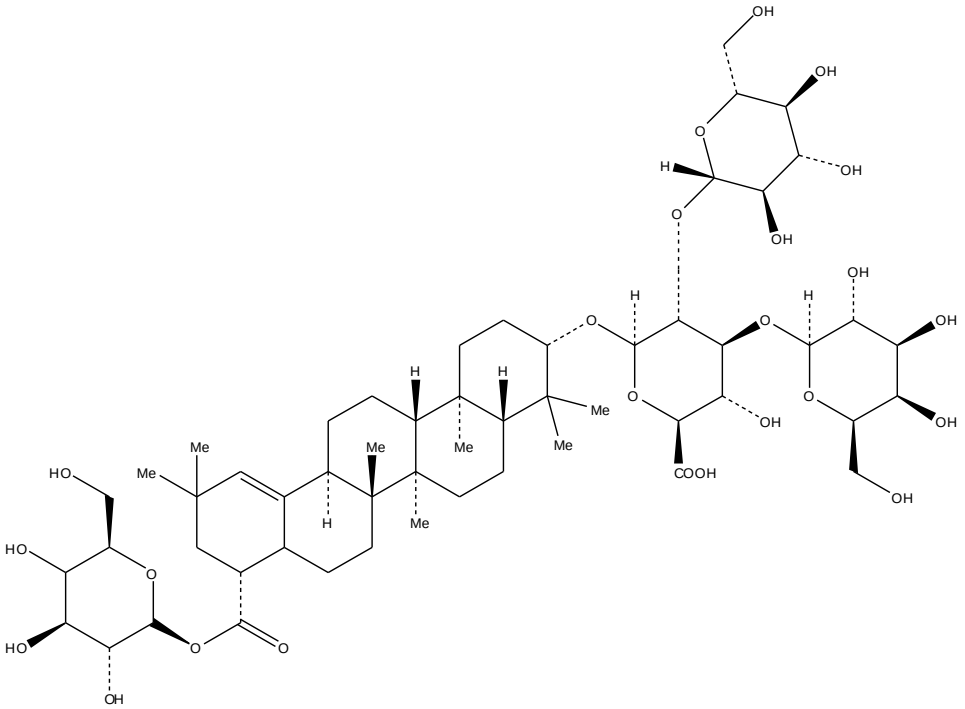
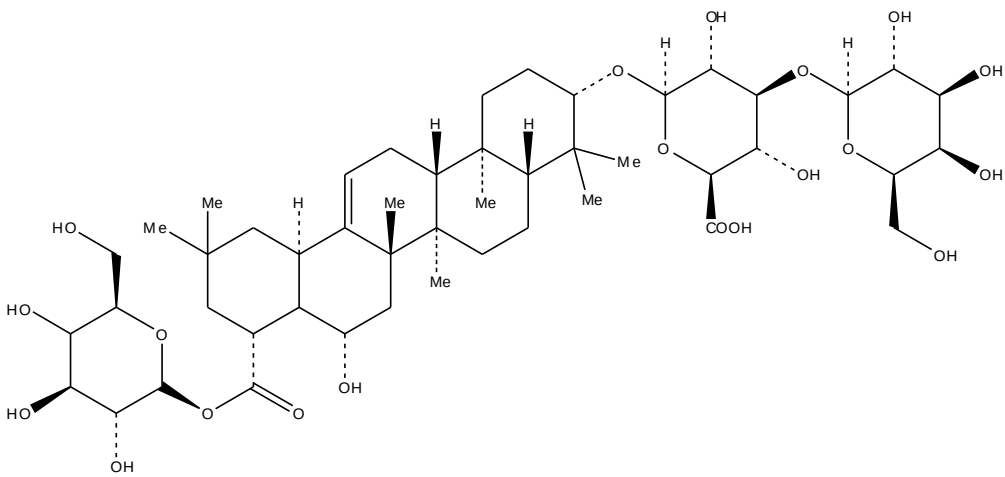


Calendasaponin A



Calendasaponin B

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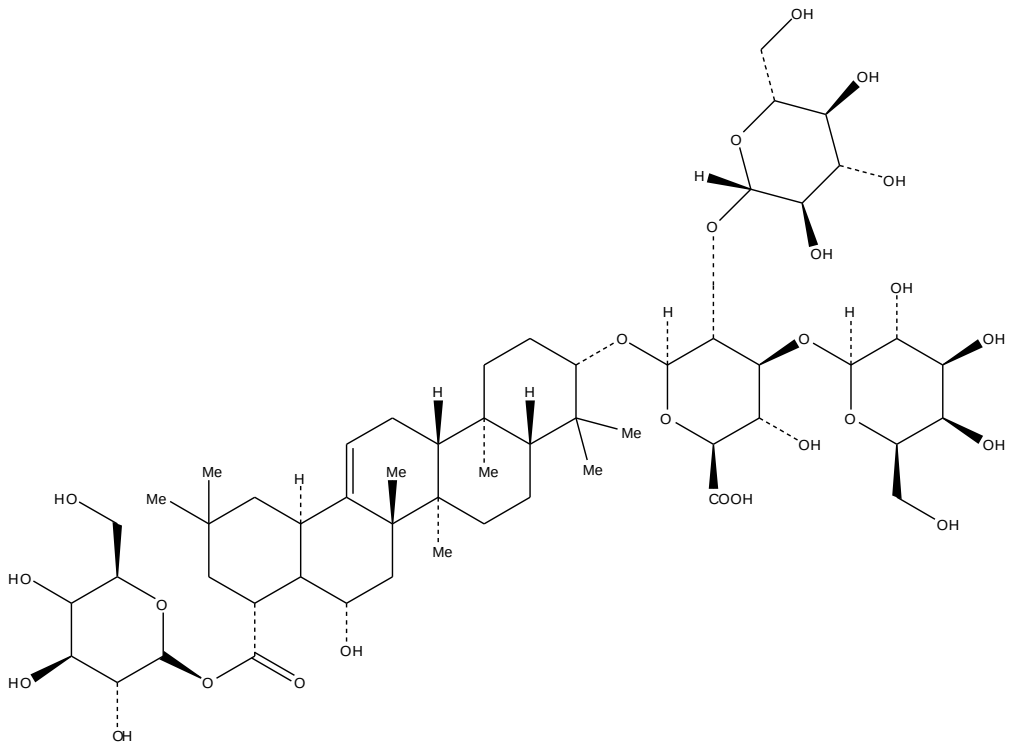
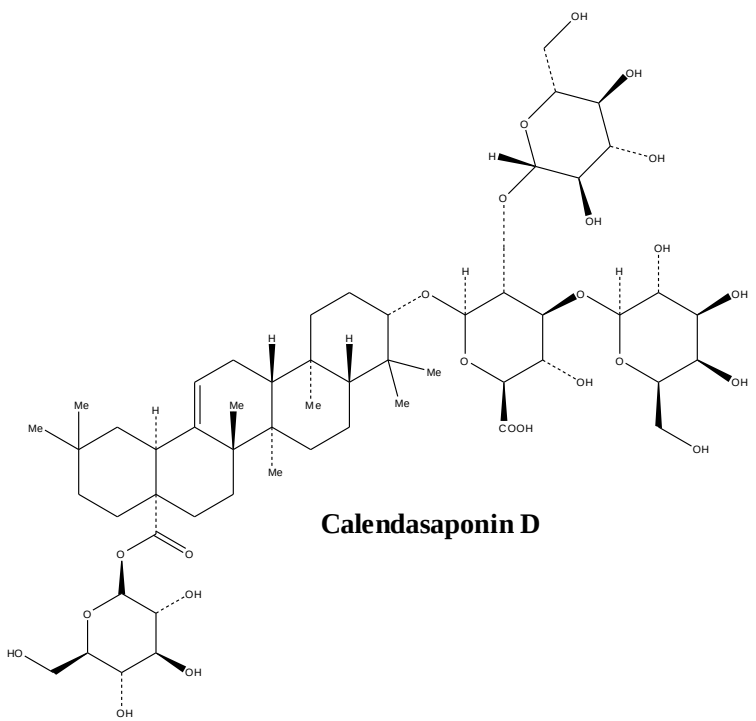


Calendasaponin C



Calendasaponin D

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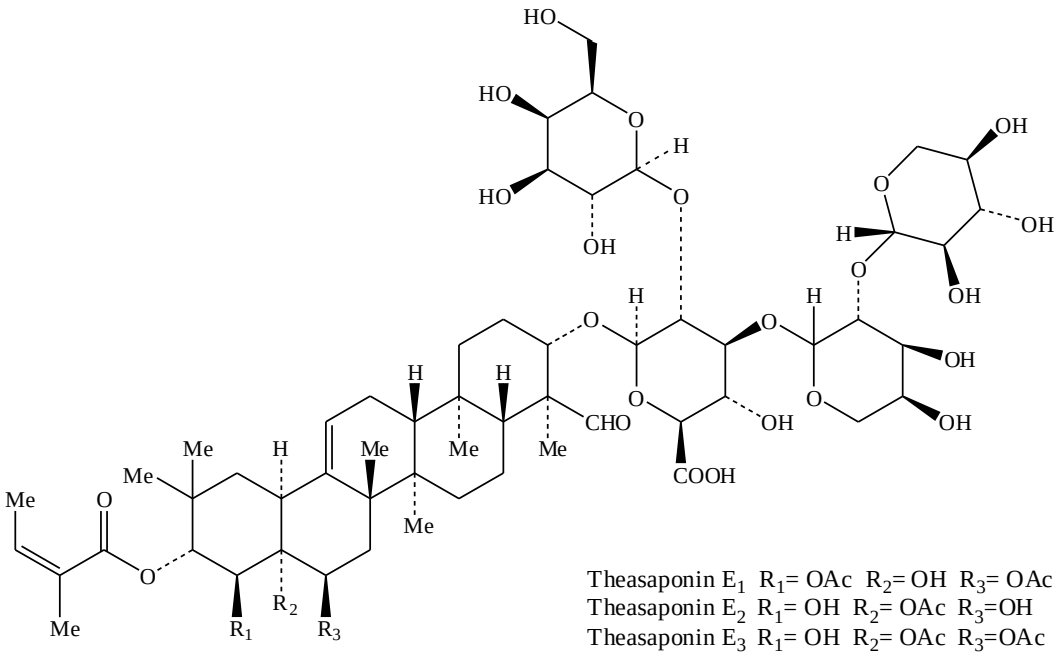
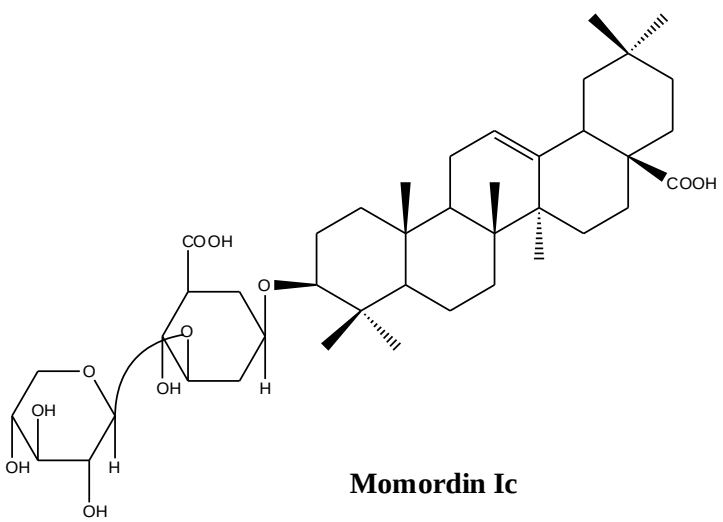


Theasaponins



Momordin Ic

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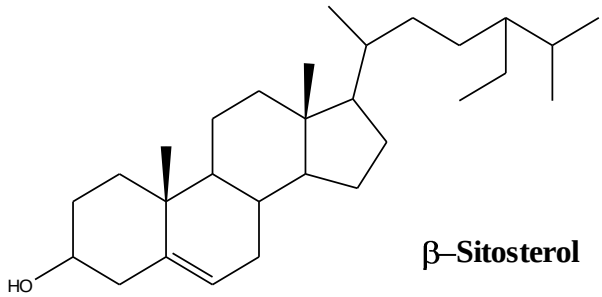
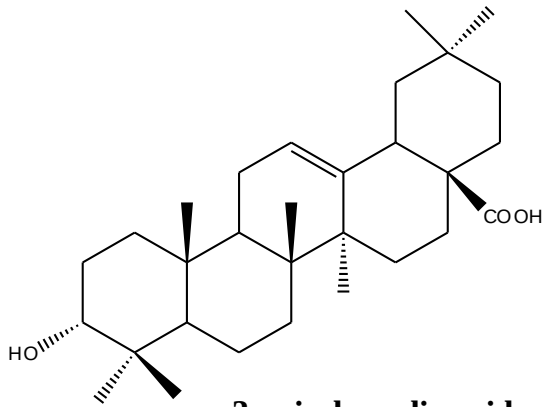
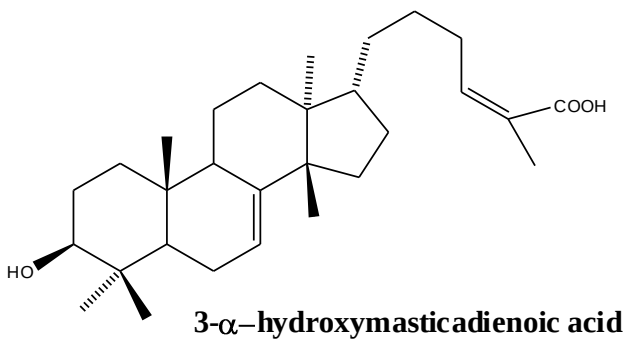


β–Sitosterol



3-epi-oleanolic acid



3-α–hydroxymasticadienoic acid

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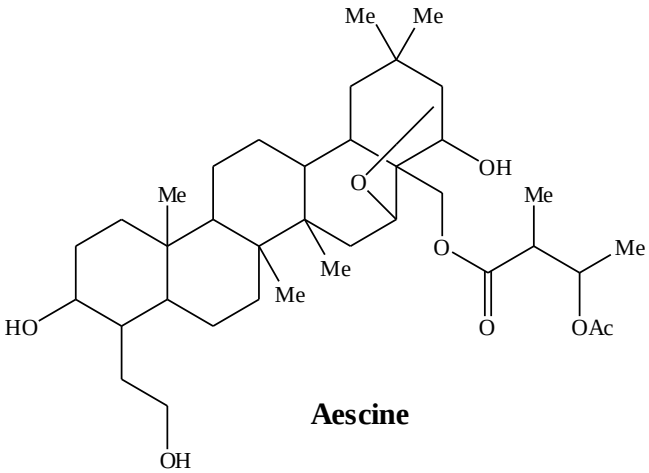
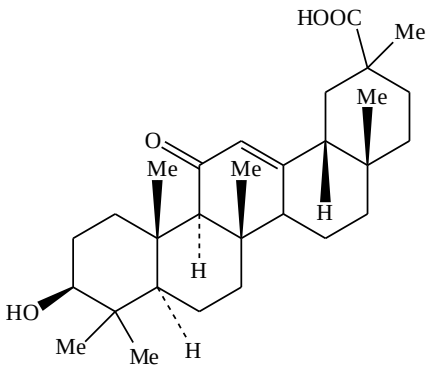
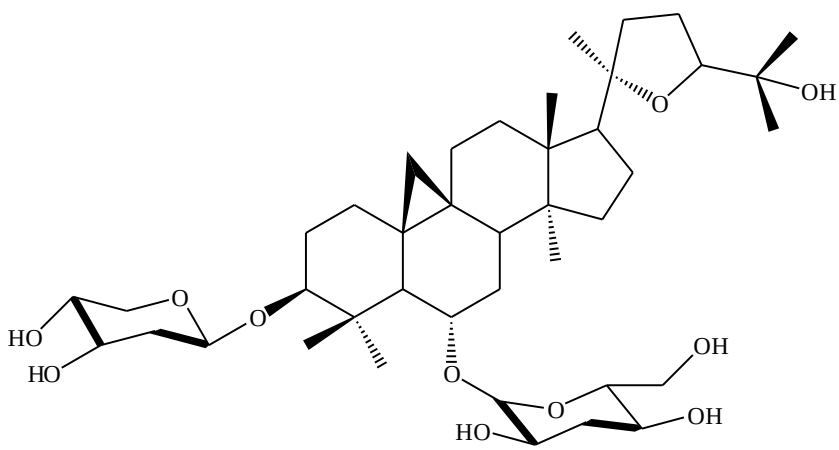


Aescine



18-β–glycyrretic acid (enoxolone)



Astragaloside IV

Figure 4. Triterpenoids with antiulcer activity.

More about miscellaneous triterpenes with gastroprotective activities are Calendasaponins A, B, C and D, triterpene oligoglycosides compounds which have induced gastric emptying in mice, and decrement in the lesions induced by ethanol- and indomethacin administration [136].

Theasaponin A_2, a triterpene saponin isolated from the saponin fraction of the seeds of *Camellia sinensis* showed inhibitory effect on ethanol-induced gastric mucosa lesions in rats. Furthermore, structure-activity relationships for theasaponins on ethanol-induced gastroprotective activities may suggest that the 28-acetyl moiety enhances activity and theasaponins having a 23-aldehyde group exhibit more potent activities [137]. Furthermore, theasaponin E1, E2 and E5 showed gastroprotective properties and the structure-activity requirements suggested that 21- and/or 22-acyl groups and acetylation contribute to their gastroprotective activity [138].

A cycloartane-type triterpene glycoside, Astragaloside IV, is the active constituent of *Astragalus* species [139] and has exhibited gastroprotective activity in the experimental model of ethanol-induce gastric lesions in the rat [140]. Furthermore, 3α-hydroxymasticadienonic acid, 3-epi-oleanolic acid and β-sitosterol are the gastroprotective compounds of *Amphipterygium adstringens*; where first and second are triterpenes. Masticadienonic acid was also isolated form the active fraction, but it was unable to inhibit ethanol-induced gastric lesions. Interestingly masticadienonic acid does not possess a hydroxyl group in the position C-3 [141]. Then, triterpenoid compounds isolated from natural products have shown gastroprotective activity, the study of those compounds may help the understanding of how natural products exert pharmacological effects.

5.3. Gastroprotective Mechanisms Described for Triterpenoids

It has been described that triterpernoids possess pharmacological activity, being their gastroprotective effect one of them. The knowledge of how those compounds exert their protective activity may provide information of their pharmacological mechanism involved.

At this moment it has been elucidated some mechanism of how triterpenoids induce their gastroprotective activity. Most of the studies have been focus on the role of prostaglandins, nitric oxide, sulfhydryls and capsaicin-sensitive neurons in the gastroprotective effect of several kinds of triterpenoids.

The antiulcerogenic effect of carbenoxolone has been attributed to the stimulation of gastric mucus production [142]; further evidence showed that carbenoxolone increases PGE_2 synthesis [143]. More recently it has been found that NO contributes to the gastroprotective effect of carbenoxolone [144]. Moreover partial participation of sulfhydryl groups has been implicated on its mechanism [140].

Besides, it was explored the gastroprotective mechanism of Astragaloside IV, where the NO synthesis is involved on its antiulcerogenic effect, while prostaglandin inhibition or endogenous sulfhydryls are not involved on its mechanism of gastroprotection [140]. More evidence shows that Astragaloside IV decreases adhesive molecules such as VCAM and E-selectin; then decreases leukocyte adherence in a model of LPS-induced inflammation. Due gastric injury is a consequence of inflammatory process; it should be interesting measure the

expression of those adhesive molecules in gastric tissue and study leukocyte adherence on gastric microcirculation. In addition, TNF-α levels, an inflammatory mediator, was decreased after Astragaloside IV administration, this event may play a role on its gastric safety [145].

Gastroprotective Triterpenoids

Triterpenoid	*Plant*	*Physiologic response*
3-*O*-acetyl aleuritolic acid	*Croton cajuara*	Reduction of gastrointestinal transit in mice
	Jatropha isabelli	Gastroprotective activity in the HCl/EtOH induce gastric lesions in mice
Boswellic acid	*Boswellia serrata*	Antiulcer activity
Camellioside A and B	*Camellia japonica*	Decrement on the lesions induce by ethanol and indomethacin
Araloside A	*Aralia elata*	Reduces gastric lesions
Calendasaponins A, B, C and D		Decrement in the lesions induced by ethanol- and indomethacin administration
Theasaponin A2, E1, E2 and E5	*Camellia sinensis*	Decrement in the lesions induced by ethanol administration
Astragaloside IV	*Astragalus* species	Gastroprotective activity in the experimental model of ethanol-induce gastric lesions in the rat
3α-hydroxymasticadienonic acid 3-epi-oleanolic acid	*Amphipterygium adstringens*	Decrement in the lesions induced by ethanol administration
Carbenoxolone	*Glycyrrhiza glaba*	Decrement in the lesions induced by ethanol administration
Aescine	*Aesculus hippocastanum*	Antisecretory mechanism, improves gastric blood flow

Another triterpenes that would decrease leukocyte adherence are boswellic acid, isolated from *Boswellia serrata*. This asseveration is done due to leukocyte adherence is induce by LTB_4 synthesis; while boswellic acid inhibit LTB_4 synthesis, being this one of the mechanism of its gastric safety [146, 147].

More evidence about gastroprotective mechanism on triterpenoid activity was elucidated when NEM (*N*-ethylmaleimide) abolished the gastroprotective effect of 3α-hydroxymasticadienonic acid in the experimental model of ethanol-induced gastric injury. This exhibits the role of endogenous sulfhydryls in the mechanism of triterpenoids [141].

Besides, Momordin Ic, an oleanolic acid oligoglycoside inhibits gastric emptying in ethanol-induced gastric lesions in mice [148]. Furthermore, this compound inhibited

indomethacin induced gastric damage in rats [149]. The gastroprotective mechanism of momordin Ic is by CPSN (capsaicin-sensitive sensory nerves) role and endogenous PGs, NO and SHs participated in this mechanism [150]. Aescine a mixture of triterpene glycosides, exerts gastroprotective properties by an antisecretory mechanism which does not involve prostaglandin synthesis however, aescine improves gastric blood flow an important factor in gastroprotective substances [151].

Furthermore, the gastroprotective mechanism of β-sitosterol is related with the participation at least in part of prostaglandins, sulfhydryls, NO and capsaicin-sensitive sensory neurons (CPNS) [152].

Even though some triterpenoid gastroprotective mechanisms have been explored, there are a few of them that remain unclear. At this moment, it has not been studied the role of hydrogen sulfide, a novel gastroprotective and anti-inflammatory mediator. Furthermore, NO has been implicated in the gastroprotective mechanism of some triterpenoids, we suggest that gastric blood flow should be measured to elucidate its role. Furthermore LTB_4 needs to be measured in more triterpenoids induced gastric safety mechanism and relate it with leukocyte adherence (Figure 5). The neuronal factors do not participate in the gastroprotection of triterpenoids, or at least do not have been described for them.

[161] In addition, there is not much information about pharmacokinetic of triterpenoids, how they are absorbed in the GI tract, or if they could be bioconverted in the liver to exert their gastroprotective activity. There are some questions that remains unclear for triterpenoids, more studies should be done to explain them.

Gastroprotective Triterpenoids

Triterpenoid	*Gastroprotective mechanism*
Carbenoxolone	Stimulation of gastric mucus production Increment of PGE_2 synthesis NO contributes to the gastroprotective effect Partial participation of sulfhydryl groups on its mechanism
Astragaloside IV	NO synthesis in involved on its antiulcerogenic effect
Boswellic acid	Inhibition of LTB_4 synthesis
3α-hydroxymasticadienoic acid	Role of endogenous sulfhydryls
Momordin Ic	CPSN (capsaicin-sensitive sensory nerves) role and endogenous PGs, NO and SHs participate in this mechanism
Aescine	Improvement of gastric blood flow

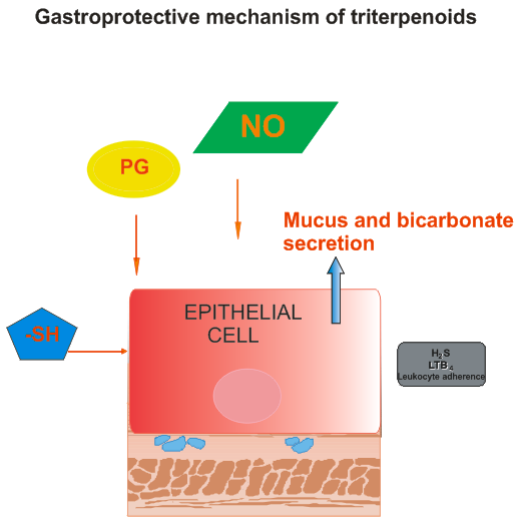


Figure 5. Mucus bicarbonate secretion and participation of NO, prostaglandins and non-proteinic sulfhydryl groups have been describe to participate in triterpenoid-induced gastroprotection mechanism. Hydrogen sulfide (H_2S), LTB_4 and leukocyte adherence remain unclear.

6. Summary and Conclusions

In summary there are several mechanisms that mediate gastric injury, all of them work together. PGs are the basis for mucosa gastric defense, they regulate gastric blood flow and gastric mucus secretion and bicarbonate throw the activation of COX-1; moreover, PGs regulate leukocyte adherence and reepithelization with COX-2. However, when both cyclooxygenases are inhibited another mechanism emerges to compensate this lack in PGs synthesis. NO and H_2S are gaseous mediators and it has been elucidated their role in gastric mucosa defense. Both gases could be synthesized to reduce the damage caused by PGs inhibition; they share roles with PGs such as the increment in gastric blood flow and the inhibition on leukocyte adherence.

Ethanol- and NSAIDs-induced gastric injuries are the most common experimental models for the study of gastroprotective drugs. Ethanol induces mucosa necrosis and NSAIDs inhibit PGs synthesis to stimulate damage. Furthermore, both increment TNF-α to induce injury. Several natural products have exhibited a significant contribution in the field of gastroprotective substances. Triterpenes are compounds with gastroprotective properties; they play this role by PGs, NO and/or -SH stimulation. There are many reports about the role that LTB_4 plays on gastric damage, however there is not much information about those levels after administration of gastroprotective triterpenoids.

Moreover, a lack on leukocyte adherence may play a role in the gastroprotective properties of some triterpenoids. Moreover, there are an unexplored field in the role of annexins, lipoxins and H_2S generation after gastroprotective triterpenoid administration. TNF-α is another inflammatory mediator that a lack on it should help to the healing of the endothelium.

In relationship with the chemical structure requirement for triterpenoids to exhibit gastroprotective properties, it has been found that a hydroxyl group in the C-3 position is related with its gastroprotective activity.

In conclusion, triterpenoid exhibit gastroprotective properties by PGs, NO and/ or -SH stimulation. There is another mechanism that remains unexplored such as H_2S generation mainly. Pharmacokinetic studies need to be done about active triterpenoids. It should help to explain its properties in the organism. Derivatives from natural triterpenes should be a strategy to improve the gastroprotective effect of this kind of natural products.

7. Acknowledgements

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Chapter 4

Biosyntheses and Bioactivities of Flavonoids in the Medicinal Plant *Scutellaria Baicalensis*

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Abstract

Scutellaria baicalensis Georgi is one of the most widely used medicinal plants, and is officially listed in the Chinese Pharmacopoeia. Its roots have been used for anti-inflammation, anticancer, decreasing blood pressures, reducing the total cholesterol level, treating bacterial and viral infections of the respiratory and the gastrointestinal tract, cleaning away heat, moistening aridity, purging fire and detoxifying toxicosis. This plant also possesses cholagogic, diuretic, and cathartic actions. Some concentrated composite herbal preparations containing *Scutellaria baicalensis* Georgi as a major ingredient in their prescriptions are widely used in oriental countries. *Scutellaria baicalensis* Georgi contains a variety of flavones, phenylethanoids, amino acids, sterols and essential oils. Its dried roots contain over 30 kinds of flavonoids, such as baicalin, baicalein, wogonin, wogonin 7-O-glucuronide and oroxylin A. The flavonoids are the main active components in *Scutellaria baicalensis* Georgi. This chapter provides up-to-date coverage of this class of flavonoids in regard to chemical structures, natural resources, biosyntheses, analytical methods and biological activities. Special attention is paid to both biosyntheses and biological activities including antioxidant and free radical scavenging, anti-inflammation, anticancer, antibacterium, anti-HIV, anti-hepatitis B virus, anti-respiratory syncytial virus and anti-SARS coronavirus properties. The

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structural diversity and the pronounced biological activities encountered in the flavonoids of *Scutellaria baicalensis* indicate that this class of compounds is worthy of further studies that may lead to new drug discovery. The review provides an account on our research work combined with a reference of the information obtained in both the English and Chinese literature.

Keywords: *Scutellaria baicalensis*; Flavonoid; Biosynthesis; Bioactivity

1. Introduction

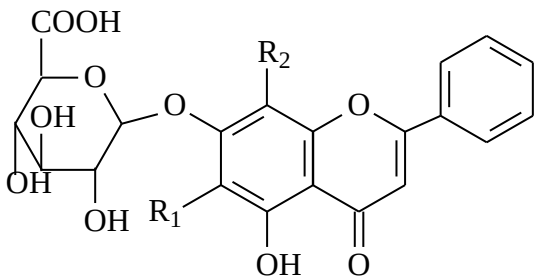
Scutellaria baicalensis (*S. baicalensis*) Georgi is one of the most widely used medicinal plants in some Asian countries, and is officially listed in the Chinese Pharmacopoeia. Its roots have been used for anti-inflammation, anticancer, decreasing blood pressures, reducing the total cholesterol level, treating bacterial and viral infections of the respiratory and the gastrointestinal tract, cleaning away heat, moistening aridity, purging fire and detoxifying toxicosis. This plant also possesses cholagogic, diuretic, and cathartic actions. Some concentrated composite herbal preparations that contain *S. baicalensis* Georgi as a major ingredient in their prescriptions are widely used in oriental countries [Lu, Jiang & Chen, 2003; Ministry of Health, 1995; Tang & Eisenbrand, 1992; Zhang et al., 2003]. *S. baicalensis* Georgi contains a variety of flavones, phenylethanoids, amino acids, sterols and essential oils, but studies on its chemical components and biological activities have been mainly confined to the flavones because they are the main active components in *S. baicalensis* Georgi [Nishikawa et al., 1999; Shen, 2000; Sichuan College of Medical Sciences, 1979; Zheng, Dong & She, 1998]. This chapter provides up-to-date coverage of this class of flavonoids in regard to chemical structures, natural resources, biosyntheses, analytical methods and biological activities, and special attention is paid to both biosyntheses and biological activities. The review provides an account on our research work combined with a reference of the information obtained in both the English and Chinese literature.

2. Chemical Structures

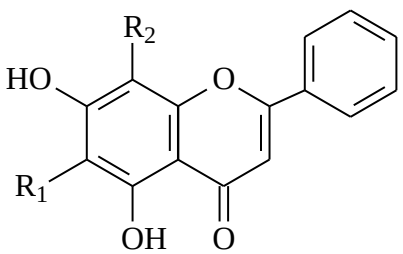
The dried roots of *S. baicalensis* Georgi contain over 30 kinds of flavonoids [Tang & Eisenbrand, 1992]. In recent years, several minor new flavonoids (2S)-5, 7, 2', 5'-tetrahydroxyflavanone, (2S)-5, 7, 2', 5'-tetrahydroxyflavanone 7-O-beta-D-glucopyranoside and 6, 2'-dihydroxy-5, 7, 8, 6'-tetramethoxyflavone were also separated and identified from *S. baicalensis* Georgi [Wang et al., 2002; Yin, 2006]. Baicalin, baicalein, wogonin, wogonin 7-O-glucuronide, oroxylin A, and oroxylin A 7-O-glucuronide are the main active components in *S. baicalensis* Georgi [Li, Jiang & Chen, 2004; Shen, 2000; Zheng, Dong & She, 1998], and their chemical structures are shown in Figure 1.

3. Natural Resources

Scutellaria baicalensis Georgi (Huangqin in Chinese) is the most widely used in China and in several oriental countries. The other *Scutellaria* species used in the traditional Chinese medicines are *Scutellaria viscidula* Bge, *Scutellaria amoena* C.H., *Scutellaria rehderiana* Diels, *Scutellaria ikonnikovi* Juz, *Scutellaria likiangensis* Diels, and *Scutellaria hypericifolia* Levl. Most flavonoids reported in *S. baicalensis* Georgi were also found in other *Scutellaria* species. More than 60 flavonoids have been identified from different sources of *Scutellaria* [Arfan et al., 2003; Li-Weber, 2009; Stutte, Eraso & Rimando, 2008; Zhang et al., 2005]. In addition, *Oroxylum indicum* contained also baicalein and baicalin [Chen, Games & Jones, 2003; Roy et al., 2007; Sun, Sun & Liu, 2006].



Baicalin (R_1=OH, R_2=H)
Wogonin 7-O-glucuronide (R_1=H, R_2=OMe)
Oroxylin A 7-O-glucuronide (R_1=OMe, R_2=H)



Baicalein (R_1=OH, R_2=H)
Wogonin (R_1=H, R_2=OMe)
Oroxylin A (R_1=OMe, R_2=H)

Figure 1. Chemical structures of six *S. baicalensis* active components [Li, Jiang & Chen, 2004].

4. Biosyntheses

Biosyntheses of the flavonoids from *S. baicalensis* Georgi were carried out mostly using cells or organs culture system. The strategies used for improving the flavonoids production

efficiency include media optimization, biotransformation, elicitation, Agrobacterium transformation and scale-up [Cole, Saxena & Murch, 2007; Matkowski, 2008]. A self-organizing fuzzy logic controller using a genetic algorithm is described, which controlled the glucose concentration for the enhancement of flavonoid production in a fed-batch cultivation of *S. baicalensis* plant cells. The substrate feeding strategy in a fed-batch culture was to increase the flavonoid production by using the proposed kinetic model. For the two-stage culture, the substrate feeding strategy consisted of a first period with 28 g/L of glucose to promote cell growth, followed by a second period with 5 g/L of glucose to promote flavonoid production. A simple fuzzy logic controller and the self-organizing fuzzy logic controller using a genetic algorithm were constructed to control the glucose concentration in a fed-batch culture. The designed fuzzy logic controllers were applied to maintain the glucose concentration at given set-points of the two-stage culture in fed-batch cultivation. The experimental results showed that the self-organizing fuzzy logic controller improved the controller's performance, compared with that of the simple fuzzy logic controller. The specific production yield and productivity of flavonoids in the two-stage culture were higher than those in the batch culture [Choi et al., 2001]. To enhance the production of baicalin and wogonin-7-O-glucuronic acid, a multilayer perceptron control system was applied to regulate the substrate feeding in a fed-batch cultivation. The optimal profile for the substrate feeding rate in a fed-batch culture of *S. baicalensis* was determined by simulating a kinetic model using a genetic algorithm. Process variable profiles were then prepared for the construction of a multilayer perceptron controller that included massive parallelism, trainability, and fault tolerance. An error back-propagation algorithm was applied to train the multiplayer perceptron. The experimental results showed that neurocontrol incorporated with a genetic algorithm improved the flavonoid production compared with a simple fuzzy logic control system. Furthermore, the specific production yield and flavonoid productivity also increased [Choi et al., 2002].

Agrobacterium rhizogenes LBA 9402 transformed root cultures of *S. baicalensis* Georgi were established and examined in respect to their capability to produce flavonoids characteristic of roots of the intact plant. An effect of a nutrient medium composition on growth and flavonoid content in the cultures was studied. Optimum for flavonoid production was half-strength Gamborg B5 medium containing 5-7% sucrose. The roots grown in the medium yielded up to 7% baicalein, 1.9 % wogonin and 1.3 % oroxylin A based on dry weight [Stojakowska & Malarz, 2000]. In another study, the composition and content of flavones were estimated in pRi T-DNA-transformed *S. baicalensis* roots obtained by the inoculation of axenically grown seedlings with a wild A4 strain of the soil bacterium Agrobacterium rhizogenes. The results obtained showed that the cultured roots contained similar basic flavones as intact roots of this plant species, i.e., baicalein and wogonin and corresponding glucuronides, baicalin and wogonoside. The content of these flavones in cultured roots was threefold lower than in the roots of intact five-year-old plants. When the roots were cultured on B5 or Murashige and Skoog medium, the ratios between major flavones changed but their total content remained unchanged. The treatment of three-week-old cultured roots with methyl ether of jasmonic acid doubled the total concentration of major flavones in roots, and the content of aglycons, baicalein and wogonin, increased to a greater degree, e.g., by 2.3 and 3.3 times, respectively. The induction of flavone production by

elicitors indicated that flavones behaved as phytoanticipins because major flavones of *S. baicalensis* manifested a distinct antimicrobial activity. The results of the short-term treatment of *S. baicalensis* roots with methyl ether of jasmonic acid showed that stress biotic factors could considerably increase the content of physiologically active flavones [Kuzovkina et al., 2005]. In addition, a transformed hairy root clone of *S. baicalensis* was established following infection with Agrobacterium rhizogenes ATCC15834. Three root clones of *S. baicalensis* were obtained, and the most active strain-the SR-03 clone was examined for its growth and baicalin content under various culture conditions. The root growth and baicalin content were maximized in a Schenk and Hildebrandt medium supplemented with 4 and 6% sucrose, respectively. The accumulation of baicalin in transformed hairy roots was enhanced through exposure to various elicitors methyl jasmonate, salicylic acid, and various concentrations of fungal cell wall. The accumulation of baicalin in the elicited cultures ranged from 10.5 to 18.3 mg/g dry weight of the roots, which was 1.5- to 3-fold the amount attained in controls [Hwang, 2006].

Using different explants of in vitro seed grown *S. baicalensis* Georgi plantlets, hairy roots were induced following inoculation of Agrobacterium rhizogenes strains A(4)GUS, R1000 LBA 9402 and ATCC11325. The A(4)GUS proved to be more competent than other strains and the highest transformation rates were observed in cotyledonary leaf explant (42.6%). The transformed roots appeared after 15-20 d of incubation on hormone free Murashige and Skoog medium. The results obtained by PCR, Southern hybridization and RT-PCR confirmed integration and expression of left and right termini-linked Ri T-DNA fragment of the Ri plasmid from A(4)GUS into the genome of *S. baicalensis* hairy roots. All the clones showed higher growth rate than non-transformed root and accumulated considerable amounts of the root-specific flavonoids. Baicalin content was 14.1-30.0% of dry root mass which was significantly higher than that of control field grown roots (18%). The wogonin content varied from 0.08 to 0.18 % among the hairy root clones which was also higher than in non-transformed roots (0.07 %) [Tiwari et al., 2008]. An approach of combining flow cytometry analysis with morphological and chemical profiling was used to assess the genetic stability and bioactive compound diversity in a *S. baicalensis* Georgi germplasm collection that was clonally maintained in vitro for a period of over 6 years. Germplasm lines, acclimatized to ex vitro conditions, exhibited distinctive plant growth and bioactive compound production capacities. The high level of genetic stability observed in in vitro maintained *S. baicalensis* lines opens up a variety of opportunities such as allowing long-term aseptic preservation and easy distribution of well-characterized germplasm lines of this medicinal plant species. This study represents a novel approach for continuous maintenance, monitoring, and production of medicinal plant tissues with specific chemistry [Alan et al., 2007].

5. Analytical Methods

The rapid qualitative and quantitative analyses of structurally closely related compounds have been an important issue of medicinal chemistry. The active components must be extracted from plant or raw medical material samples prior to analysis. Several extraction

techniques such as solid-phase extraction, supercritical fluid extraction and pressurized hot water extraction have been developed for the extraction of the bioactive components from *S. baicalensis* [Lin, Tsai & Wen, 1999; Ong & Len, 2003; Zgorka & Hajnos, 2003]. Solid-phase extraction is used to selectively remove interfering matrix components and improved assay selectivity, accuracy, and sensitivity. A solid-phase extraction method was recently developed for simultaneous extraction of flavanes (baicalin, baicalein, chrysin, scutellarein) and some phenolic acids in aerial and underground parts of *S. baicalensis* Georgi [Zgorka & Hajnos, 2003]. The application of optimized enrichment conditions, elaborated on octadecyl and quaternary amine BakerBond microcolumns, led to the extraction of both groups of analytes with recoveries > 95% and variation coefficients < 5%. Supercritical fluid extraction is another widely used technique for extraction of active components from plant or raw medical material samples, in which supercritical carbon dioxide is often used as an extraction solvent. For the extraction of polar or ionic compounds, organic solvents are added as modifiers or the compounds are first derivatized to decrease their polarity. Supercritical fluid extraction was applied to the extraction of baicalin, baicalein and wogonin from *S. baicalensis*, and gave higher yields of the three flavanoids in shorter time than ultrasonic or percolation extraction [Lin, Tsai & Wen, 1999]. Pressurized liquid extraction with methanol as solvent was also proposed for the extraction of baicalein from Scutellariae radix [Ong & Len, 2003]. The comparable performance of pressurized liquid extraction with reference to Soxhlet extraction was due to the higher diffusion rate and higher solubility of analyte in the solvent as a result of the higher temperature. To reduce the use of organic solvent, pressurized hot water extraction was developed for the extraction of baicalein from Scutellariae radix [Ong & Len, 2003]. Although baicalein was insoluble in water, the results showed that water with a small proportion (20%) of ethanol as organic modifier at a temperature below its boiling point and a small applied pressure was able to extract an equivalent amount of baicalein from medicinal plant compared with Soxhlet extraction with aqueous organic solvent. The results obtained by pressurized hot water extraction were in agreement with those using pressurized liquid extraction with methanol as the extraction solvent.

High-performance liquid chromatographic methods have been widely applied to the separation and determination of *S. baicalensis* active components in various matrices including plant, raw medical material, medicinal preparations and biological fluid samples. For example, baicalin, baicalein and wogonin in Scutellariae radix were determined by HPLC on a ODS Hypersil column with gradient elution of acetonitrile and 0.1 M H_3PO_4 as mobile phase and detection at 280 nm [Rhee et al., 1997]. The six main bioactive components, baicalein, baicalin, wogonin, wogonin glucuronide, oroxylin-A and oroxylin-A glucuronide in Scutellariae radix could be determined simultaneously by ion-pair high-performance liquid chromatography on a stainless-steel column packed with TSK gel LS-410 with aqueous 32% acetonitrile, containing 5 mM tetrapentylammonium bromide, as mobile phase, adjusted to pH 4 with H_3PO_4 [Sagara et al., 1985]. In another study, flavonoid constituents of the roots of *S. baicalensis* Georgi were determined by HPLC on a column of Develosil ODS-5 at 50 °C, with 274 nm detection and tetrahydrofuran-dioxan-methanol-acetic acid-5% H_3PO_4-H_2O (145:125:50:20:2:322) or tetrahydrofuran-acetic acid -5% H_3PO_4-H_2O (95:10:1:444) as mobile phase [Tomimori et al., 1985]. By a combination of two mobile phases, total eleven

flavonoids were separated in two runs. In addition, the HPLC method has been used for quality control of medical products based on *S. baicalensis* Georgi by the determination of baicalin content in Pharmacopoeia of the People's Republic of China [Ministry of Health, 1995].

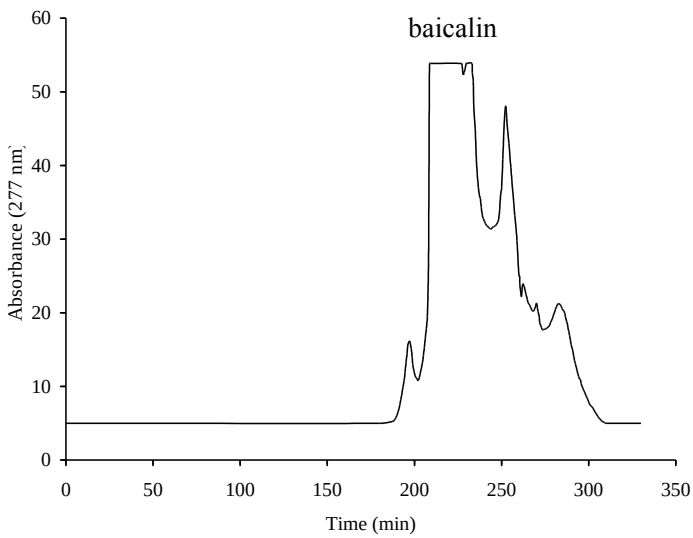
High-speed counter-current chromatography (HSCCC) is a relatively new, all-liquid separation technique. Because there is no solid support matrix in HSCCC column, it eliminates irreversible adsorptive loss, denaturation and contamination of samples from the solid support matrix used in the conventional chromatographic column. The method has been successfully applied to the analysis and separation of various natural products [Li & Chen, 2008]. Baicalin could be separated and purified from *S. baicalensis* Georgi by HSCCC [Lu, Jiang & Chen, 2003]. The separation was performed in two steps with a two-phase solvent system composed of *n*-butanol-water (1: 1), in which the lower phase was used as the mobile phase at a flow-rate of 1.0 ml/min in the head-to-tail elution mode. A total of 37.0 mg of baicalin at 96.5% purity was yielded from 200 mg of the crude baicalin (containing 21.6% baicalin) with 86.0% recovery. The HSCCC chromatogram is shown in Figure 2. In order to isolate baicalein, wogonin and oroxylin A from the same herb, a HSCCC method with a two-phase solvent system composed of *n*-hexane–ethyl acetate–*n*-butanol–water (1:1:8:10, v/v) was developed by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.0 ml/min after 4 h (Figure 3). The method yielded 144.8 mg of baicalein in 95.7% purity, 50.2 mg of wogonin in 98.5% purity, and 12.4 mg of oroxylin A in 93.2% purity from 500 mg of the crude extract in a one-step separation. The recoveries of baicalein, wogonin and oroxylin A were 92.7%, 91.6% and 92.5%, respectively [Li & Chen, 2005]. In the future, bioactive components at highly purity will be used instead of crude extracts in the medicinal preparations of *S. baicalensis*. Therefore, separation and purification techniques will play an important role in these studies, and high-speed counter-current chromatography will be more widely used for the preparative separation and purification of *S. baicalensis* active components on a large scale.

Although gas chromatography was not widely employed for the determination of flavonoids in *S. baicalensis* because of their high polarity, low volatility and poor thermal stability, thin layer chromatography, capillary electrophoresis and micellar electrokinetic capillary chromatography have been used for the determination of *S. baicalensis* active components in various matrices. In the future, high-performance liquid chromatography-mass spectrometry will play a more important role in the studies of bioactive components of *S. baicalensis*. The main advantages of HPLC–MS are its high speed and sensitivity compared with other hyphenated identification techniques such as HPLC–nuclear magnetic resonance and HPLC–infrared. In most situations, HPLC–MS/MS or HPLC–$(MS)^n$ is preferred over HPLC–MS for the structure elucidation of unknown mixtures, because multistage MS can provide additional information on fragmented ions, facilitating structural assignments [Li, Jiang & Chen, 2004].

6. Biological Activities

6.1. Antioxidant and Free Radical Scavenging Effects

The extract from *S. baicalensis* inhibited lipid peroxidation caused by chromium [Sawicka et al., 2008]. In another study, the antioxidant capacities of 45 selected medicinal plants were evaluated using ferric reducing antioxidant power and Trolox equivalent antioxidant capacity assays, and the results showed that *S. baicalensis* possessed strong antioxidant capacity [Li et al., 2008]. A study examined the antioxidant activity of hexane, acetone, and methanol extracts, as well as baicalein purified from the dry roots of *S. baicalensis*, in heated canola oil. Among the three extracts, the acetone extract was the most effective against oxidation of canola oil, followed by the methanol extract of the dry roots. The antioxidant activity of these three extracts correlated well with their content of baicalein, which provided strong protection to canola oil from oxidation. The antioxidant activity of the acetone extract was dose-dependent. The acetone extract at 100 ppm or above was even more effective than butylated hydroxytoluene at 200 ppm in protecting canola oil from oxidation [Chen et al., 2000].



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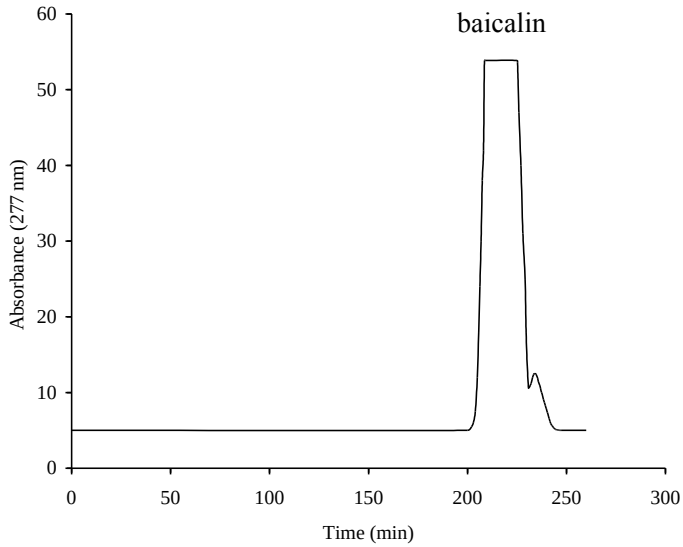

Figure 2. HSCCC chromatograms of the crude baicalin extracted from *Scutellaria baicalensis* Georgi (A) the first separation, and (B) the second separation [Lu, Jiang & Chen, 2003].

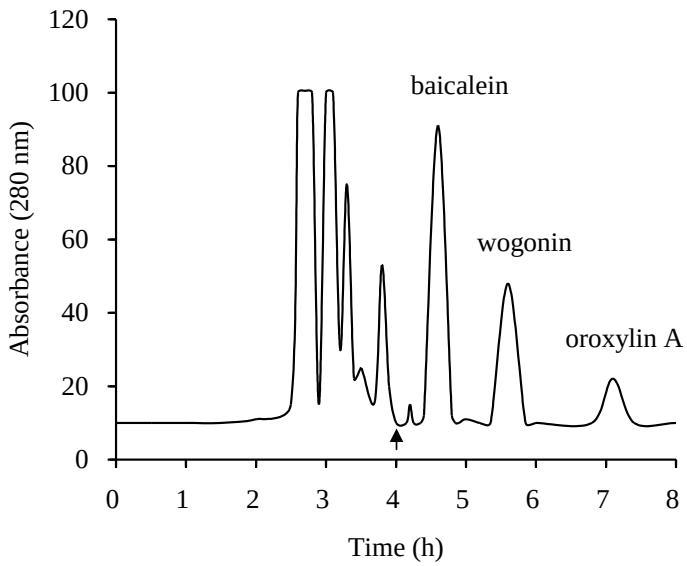

Figure 3. Chromatogram of the crude extract from *Scutellaria baicalensis* Georgi by HSCCC separation. *Conditions*: column, multilayer coil of 2.6 mm i.d. PTFE tube with a total capacity of 325 ml; rotary speed, 1000 rpm; solvent system, *n*-hexane–ethyl acetate–*n*-butanol–water (1:1:8:10, v/v/v/v); mobile phase, lower phase (water phase); flow-rate, 0–4 h, 1.0 ml min^{-1} and 4–8 h, 2.0 ml min^{-1}; detection at 280 nm; sample size, 500 mg; retention of the stationary phase, 51%. The arrow indicates the flow-rate of the mobile phase was increased stepwise from 1.0 ml min^{-1} to 2.0 ml min^{-1} after 4 h [Li & Chen, 2005].

In vitro studies using electron paramagnetic resonance spectroscopy with the spin trap 5-methoxycarbonyl-5-methyl-1-pyrroline-N-oxide revealed that baicalein scavenged superoxide but did not mimic the effects of superoxide dismutase. Baicalein could scavenge reactive oxygen species generation in cardiomyocytes, and protect against cell death in an ischemia-reperfusion model when given only at reperfusion [Shao et al., 2002]. Five main flavonoids from *S. baicalensis* were also evaluated for their scavenging abilities with DPPH radical-generating system and due to limited solubility only two flavonoids were investigated for their ability to scavenge hydroxyl radical by the aromatic hydroxylation method. The total extract was also tested in both the experimental arrangements. In experiments with DPPH, only baicalin and baicalein displayed a significant scavenging effect, while the production of hydroxyl radicals generated by UV photolysis of H_2O_2 was considerable decreased in the presence of baicalin and wogonin glucuronide. After comparison with results obtained for the total extract, it was concluded that the scavenging activity of the extract against DPPH was mainly derived from baicalein. On the other hand, baicalin, wogonin glucuronide and probably other flavonoids participated in scavenging hydroxyl radicals [Bochorakova et al., 2003].

Baicalein, oroxylin A and wogonin all exhibited significant antioxidative and free-radical scavenging activities. In respect of their nitric oxide inhibition, wogonin was superior to all the other flavonoids, while oroxylin A was the most potent in the inhibition of lipid peroxidation. Wogonin proved to be the most potent in its antiinflammatory activity against carrageenan-induced rat hind paw edema. There was a correlation between the in vivo anti-inflammatory activity and the in vitro antioxidative activities [Huang, Lee & Yang, 2006]. In a sudy, free-radical scavenging activities of baicalein, baicalin, wogonin and wogonoside were examined by electron paramagnetic resonance (EPR). The results showed that baicalein and baicalin scavenged hydroxyl radical, superoxide anion, DPPH radical and alkyl radical in a dose-dependent manner, while wogonin and wogonoside showed subtle or no effect on these radicals. Baicalein was the most effective free-radical scavenger among the four tested compounds [Gao et al., 2000]. In another study, free radical scavenging and antioxidant activities of baicalein, baicalin, wogonin and wogonoside were examined in different systems. Ten μmol/L of baicalein and baicalin effectively inhibited lipid peroxidation of rat brain cortex mitochondria induced by Fe^{2+}-ascorbic acid, AAPH or NADPH, while wogonin and wogonoside showed significant effects only on NADPH-induced lipid peroxidation. In a study on cultured human neuroblastoma SH-SY5Y cells system, it was found that 10 μmol/L of baicalein and baicalin significantly protected cells against H_2O_2-induced injury. Baicalein was the most effective antioxidant among the four tested compounds in every system due to its o-tri-hydroxyl structure in the A ring. Compared with a well-known flavonoid, quercetin, the antioxidant activity of baicalein was lower in DPPH or AAPH system, but a little higher in those systems which might associate with iron ion [Gao et al., 1999]. In addition, the combination of *Scutellaria baicalensis* and grape seed proanthocyanidins could potentially enhance their antioxidant efficacy, allowing lower dosages of each drug to be used. This had the advantage of avoiding possible side effects that might arise when higher doses of a single herb were used in an attempt to achieve a maximum degree of antioxidant activity [Shao et al., 2004].

6.2. Anti-Inflammation Effect

The anti-inflammatory effects of baicalein, baicalin and wogonin were evaluated in a murine model of acute experimental colitis induced by dextran sulfate sodium. Baicalein, but not baicalin or wogonin, given orally at 20 mg/kg for ten days, ameliorated all the considered inflammatory symptoms of the induced colitis, such as body weight loss, blood haemoglobin content, rectal bleeding and other histological and biochemical parameters. The effect of baicalein was similar to that of sulfasalazine, the reference drug given at 50 mg/kg [Hong et al., 2002]. In order to elucidate the mechanism of the antiinflammatory action of baicalein and wogonin, the effects of these compounds were investigated on lipopolysaccharide-induced nitric oxide production in a macrophage-derived cell line, RAW 264.7. Baicalein (5-25 μM) and wogonin (5-50 μM) inhibited lipopolysaccharide-induced nitric oxide generation in a concentration-dependent manner. The inhibitory effect of these compounds was observed only when they were added at the start of cell incubation soon after the stimulation with lipopolysaccharide. Baicalein (25 μM) and wogonin (25 μM) also inhibited protein expression of inducible nitric oxide synthase (iNOS). This inhibitory effect of wogonin was stronger than that of baicalein, which agreed with the result that wogonin showed stronger inhibition of nitric oxide production than baicalein. These results suggested that baicalein and wogonin attenuated lipopolysaccharide-stimulated nitric oxide synthase expression in macrophages and thus may help to explain the antiinflammatory action of these flavonoid compounds [Wakabayashi, 1999].

Baicalein and baicalin were examined for their effects on lipopolysaccharide (LPS)-induced cyclooxygenase-2 (COX-2) gene expression in Raw 264.7 macrophages. Baicalein, but not baicalin, inhibited COX-2 gene expression in LPS-induced Raw 264.7 cells. However, both polyphenolic compounds inhibited LPS-induced iNOS protein expression, iNOS mRNA expression, and NO production in a dose-dependent manner. Baicalein and baicalin had no effect on LPS-induced nuclear factor-kappa B (NF-kappa B) and cAMP response element binding protein (CREB) DNA binding activity. Baicalein, but not baicalin, significantly inhibited the DNA binding activity of CCAAT/enhancer binding protein beta (C/EBP beta). The differential effects of baicalein and baicalin on COX-2 gene expression in LPS-induced Raw 264.7 cells were mediated through inhibition of C/EBP beta DNA binding activity. Baicalein acted to inhibit inflammation through inhibition of COX-2 gene expression through blockade of C/EBP beta DNA binding activity [Woo et al., 2006]. In order to elucidate the mechanism of action of baicalin, it was tested whether could interfere with chemokines or chemokine receptors, which were critical mediators of inflammation and infection. The results showed that baicalin inhibited the binding of a number of chemokines to human leukocytes or cells transfected to express specific chemokine receptors. This was associated with a reduced capacity of the chemokines to induce cell migration. Go-injection of baicalin with CXC chemokine interleukin-8 (IL-8) into rat skin significantly inhibited IL-8 elicited neutrophil infiltration. Baicalin did not directly compete with chemokines for binding to receptors, but rather acted through its selective binding to chemokine ligands, which was supported by the fact that baicalin cross-linked to oxime resin bound chemokines of the CXC (stromal cell-derived factor (SDF)-1 alpha, IL-8), CC (macrophage inflammatory protein (MIP)-1 beta, monocyte chemotactic protein (MCP)-2), and C (lymphotactin (Ltn))

subfamilies. Baicalin did not interact with CX3C chemokine fractalkine/neurotactin or other cytokines, such as TNF-alpha and IFN-gamma, indicating that its action was selective. One possible anti-inflammatory mechanism of baicalin was to bind a variety of chemokines and limit their biological function [Li et al., 2000a].

6.3. Anticancer Effects

S. baicalensis exerted dose- and time-dependent growth inhibition to two human prostate cancer cell lines (LNCaP, androgen dependent, and PC-3, androgen independent). However, the PC-3 cells were slightly more sensitive than LNCaP cells, although the former is androgen independent. Significant reduction of prostaglandin E-2 (PGE2) synthesis in both cells after treatment with *S. baicalensis* resulted from direct inhibition of COX-2 activity rather than COX-2 protein suppression. *S. baicalensis* also inhibited prostate-specific antigen production in LNCaP cells. Finally, S. baicalensis suppressed expression of Cyclin DI in LNCaP cells, resulting in a G(1) phase arrest, while inhibiting Cdk1 expression and kinase activity in PC-3 cells, ultimately leading to a G(2)/M cell cycle arrest. Animal studies showed a 50% reduction in tumor volume after a 7-wk treatment period [Ye et al., 2007]. Furthermore, four compounds (baicalein, wogonin, neobaicalein and skullcapflavone) capable of inhibiting prostate cancer cell proliferation were separated and identified from *S. baicalensis*. Comparisons of the cellular effects induced by the entire extract versus the four-compound combination produced comparable cell cycle changes, levels of growth inhibition, and global gene expression profiles (r^2 = 0.79). Individual compounds exhibited antiandrogenic activities with reduced expression of the androgen receptor and androgen-regulated genes. In vivo, baicalein (20 mg/kg/d p.o.) reduced the growth of prostate cancer xenografts in nude mice by 55% at 2 weeks compared with placebo [Bonham et al., 2005].

In order to study anticancer activity of *S. baicalensis* on head and neck squamous cell carcinoma (HNSCC) in vitro and in vivo and to investigate its effect on COX-2, which converts arachidonic acid to PGE2 and is highly expressed in HNSCC, two human HNSCC cell lines (SCC-25 and KB) and a nontumorigenic cell line (HaCaT) were tested in vitro for growth inhibition, proliferation cell nuclear antigen expression, and COX-2 activity and expression after treatment with its extract. *S. baicalensis*, indomethacin (a nonselective COX inhibitor) and celecoxib (a selective COX-2 inhibitor) demonstrated a strong growth inhibition in both tested human HNSCC cell lines. No growth inhibition of HaCaT cells was observed with *S. baicalensis*. The IC50s were 150 μg/ml for *Scutellaria baicalensis*, 25 μM for celecoxib, and 75 μM for baicalein and indomethacin. *S. baicalensis* as well as celecoxib and indomethacin, but not baicalein, suppressed proliferation cell nuclear antigen expression and PGE2 synthesis in both cell types. *S. baicalensis* inhibited COX-2 expression, whereas celecoxib inhibited COX-2 activity directly. A 66% reduction in tumor mass was observed in the nude mice by *S. baicalensis,* which selectively and effectively inhibited cancer cell growth in vitro and in vivo and could be an effective chemotherapeutic agent for HNSCC. Inhibition of PGE2 synthesis via suppression of COX-2 expression might be responsible for its anticancer activity. Differences in biological effects of *S. baicalensis* compared with baicalein suggested the synergistic effects among components in *S. baicalensis* [Zhang et al.,

2003]. In another study, cell lines from the most common human cancers, including squamous cell carcinoma (SCC-25, KB), breast cancer (MCF-7), hepatocellular carcinoma (HepG2), prostate carcinoma (PC-3 and LNCaP), and colon cancer (KM-12 and HCT-15) were tested for anticancer activity of *S. baicalensis*. The results showed that *S. baicalensis* strongly inhibited cell growth in all cancer cell lines tested. Furthermore, prostate and breast cancer cells (PC-3, LNCaP, and MCF-7) are slightly more sensitive than other type of cancer cells. It also inhibited PGE2 production, indicating that suppression of tumor cell growth may be due to its ability to inhibit COX-2 activity [Ye et al., 2002].

Wogonin inhibited the growth and tumor angiogenesis of human gastric carcinoma in nude mice. Wogonin suppressed the vascular endothelial growth factor (VEGF)-stimulated migration and tube formation of human umbilical vein endothelial cells. It also restrained VEGF-induced tyrosine phosphorylation of vascular endothelial growth factor receptor 2. This inhibition of receptor phosphorylation was correlated with a significant decrease in VEGF-triggered phosphorylated forms of ERK, AKT and p38 [Lu et al., 2008]. In addition, effects of wogonin were examined in estrogen receptor (ER)-positive and -negative human breast cancer cells in culture for proliferation, cell cycle progression, and apoptosis. Cell growth was attenuated by wogonin (50-200 μM), independently of its ER status, in a time- and concentration-dependent manner. Apoptosis was enhanced and accompanied by upregulation of PARP and Caspase 3 cleavages as well as proapoptotic Bax protein. Akt activity was suppressed and reduced phosphorylation of its substrates, GSK-3 beta and p27, was observed. Suppression of Cyclin D1 expression suggested the downregulation of the Akt-mediated canonical Writ signaling pathway. ER expression was downregulated in ER-positive cells, while c-ErbB2 expression and its activity were suppressed in ER-negative SK-BR-3 cells. Wogonin feeding to mice showed inhibition of tumor growth of T47D and MD-AMB-231 xenografts by up to 88% without any toxicity after 4 weeks of treatment [Chung et al., 2008].

In order to compare the effect of individual botanical extracts with combinations of extracts on prostate cell viability, *S. baicalensis*, *Rabdosia rubescens*, *Panax-pseudo ginseng*, *Dendranthema morifolium*, *Glycyrrhiza uralensis* and *Serenoa repens* were tested. Each extract significantly inhibited the proliferation of prostate cell lines in a time- and dose-dependent manner except *S. repens*. The most active extracts, *S. baicalensis*, *D. morifolium*, *G. uralensis* and *R. rubescens* were tested as two-extract combinations. *S. baicalensis* and *D. morifolium* when combined were additive with a trend toward synergy, whereas *D. morifolium* and *R. rubescens* together were additive. The remaining two-extract combinations showed antagonism. The four extracts together were significantly more effective than the two-by-two combinations and the individual extracts alone. Combining the four herbal extracts significantly enhanced their activity in the cell lines tested compared with extracts alone [Adams et al., 2006].

6.4. Antibacterial Effects

Stem blister canker is a serious stem disease in the Populus genus in China, and the pathogen was *Botryosphaeria dothidea*. Seven selected plant species were extracted with the solvent 95% ethanol to yield ethanol extracts which were used to evaluate their antifungal activity against poplar stem canker pathogen by a mycelial radial growth inhibition test. The extract of *S. baicalensis* showed strong antifungal activity, and the median inhibitory concentration (IC50) was 0.9675 mg/ml [Zhou et al., 2008]. In another study, the extracts of 56 widely used dried Chinese medical plants were screened for their antimycotic properties against pathological phyla of *Aspergillus fumigatus*, *Candida albicans*, *Geotrichum candidum* and *Rhodotorula rubra*, and *S. baicalensis* had the highest activity against Candida albicans [Blaszczyk, Krzyzanowska & Lamer-Zarawska, 2000].

The antibacterial effects of water extracts of *S. baicalensis* and its major flavonoid components, baicalin and baicalein, on Salmonella typhimurium, a representative enteric pathogen, were also studied. Through a Kirby-Bauer disc analysis, the growth-inhibition activity of Scutellariae Radix against S. typhimurium was found to be compatible with commercial antibiotics, such as ampicillin, chloramphenicol, and streptomycin. In contrast, the growth of a nonpathogenic E. coli strain was unaffected by Scutellariae Radix. To examine the effect of polyphosphate kinase (ppk), a putative virulence factor, on the antibacterial activity of Scutellariae Radix, the growth profile of a ppk mutant of S. typhimurium was investigated in a tryptic soy broth containing different concentrations of water extracts of Scutellariae Radix. The ppk mutant was able to grow in 6 mg/ml of water extracts of Scutellariae Radix, whereas the wild-type could not, implying that the inactivation of ppk made S. typhimurium more resistant to the antibacterial activity of Scutellariae Radix. No enhanced resistance was observed in a ppk mutant of S. typhimurium complemented with a ppk expression vector. The attenuation of the virulence by ppk inactivation was also observed in a virulence assay using BALB/c mice. Neither baicalin nor baicalein exhibited any growth-inhibition activity against S. typhimurium. The water extracts of Scutellariae Radix stimulated the transcription of ppk, especially in the early growth-stage of S. typhimurium [Hahm et al., 2001].

6.5. Antivirus Effects

Extracts of *S. baicalensis* displayed a wide spectrum of antiviral activity [Blach-Olszewska et al., 2008]. To study the effect of *S. baicalensis* extracts on interferons (IFNs), tumor necrosis factor alpha (TNF-alpha), and interleukin (IL) production and virus replication, uninfected and vesicular stomatitis virus (VSV)-infected human peripheral blood leukocytes (PBLs) were used. The results indicated that baicalein- and wogonin-containing extracts modulate cytokine production, which inhibited IFN-alpha and IFN-gamma and stimulated TNF-alpha and IL (IL-12, IL-10) production. They also augmented the resistance of PBLs to VSV. In another study, forty-four medicinal herbs were tested for antiviral activities against respiratory syncytial virus (RSV) by means of the cytopathologic effect (CPE) assay. Twenty-seven of the 44 medicinal herbs showed potent or moderate antiviral

activities against RSV with 50% inhibition concentration (IC50) ranging from 6.3 to 52.1 μg/ml, and with selectivity index (SI) ranging from 2.0 to 32.1. Further purification of the active extracts from *S. baicalensis* Georgi led to the identification of wogonin and oroxylin A as the potent anti-RSV components [Ma et al., 2002]. In addition, commercial antiviral agents and pure chemical compounds extracted from traditional Chinese medicinal herbs were screened against 10 clinical isolates of severe acute respiratory syndrome (SARS) coronavirus by neutralisation tests with confirmation by plaque reduction assays, and baicalin showed strong antiviral activity [Chen et al., 2004].

The aqueous and methanol extracts of thirty-one herbs traditionally used as anti-fever remedies in China were screened for their in vitro inhibition on human immunodeficiency virus type-1 (HIV-1) protease. The aqueous extracts of *S. baicalensis* elicited significant inhibition (>90%) at a concentration of 200 μg/ml [Lam et al., 2000]. Baicalin at the noncytotoxic concentration inhibited both T cell tropic (X4) and monocyte tropic (R5) HIV-1 Env protein mediated fusion with cells expressing CD4/CXCR4 or CD4/CCR5. Furthermore, presence of baicalin at the initial stage of HIV-1 viral adsorption blocked the replication of HIV-1 early strong stop DNA in cells. Since baicalin did not inhibit binding of HIV-1 gp120 to CD4, it might interfere with the interaction of HIV-1 Env with chemokine coreceptors and block HIV-1 entry of target cells [Li et al., 2000b]. Anti-HIV activities of *S. baicalensis*, baicalein and baicalin have been emphasized in review paper [Wu et al., 2001].

By using an hepatitis B virus (HBV)-producing cell line in vitro culture system, wogonin could suppress HBV surface antigen production ($P < 0.001$) without evidence of cytotoxicity. By assaying the endogenous HBV DNA polymerase activity, both the relaxed circular and the linear forms of HBV DNA were significantly reduced in the wogonin-treated group [Huang et al., 2000]. In another study, wogonin's anti-HBV activity both in vitro and in vivo was investigated. In the human HBV-transfected liver cell line HepG2.2.15, wogonin effectively suppressed the secretion of the HBV antigens with an IC50 of 4 μg/ml at day 9 for both HBsAg and HBeAg. Consistent with the HBV antigen reduction, wogonin also reduced HBV DNA level in a dose-dependent manner. Duck hepatitis B virus (DHBV) DNA polymerase was dramatically inhibited by wogonin with an IC50 of 0.57 μg/ml. In DHBV-infected ducks wogonin dosed i.v. once a day for 10 days reduced plasma DHBV DNA level with an ED50 of 5 mg/kg. The in vivo anti-HBV effect of wogonin in ducks was confirmed by Southern blotting of DHBV DNA in the liver. Histopathological evaluation of the liver revealed significant improvement by wogonin. In addition, in human HBV-transgenic mice, wogonin dosed i.v. once a day for 10 days significantly reduced plasma HBsAg level. Immunohistological staining of the liver confirmed the HBsAg reduction by wogonin. This suggested that wogonin possessed potent anti-HBV activity both in vitro and in vivo [Guo et al., 2007].

Table 1. Bioactivities of baicalin

Bioactivities	References
Antioxidant activity	[Broncel et al., 2007; Chen, Nishida & Konishi, 2003; Kim, 2005]
Anti-inflammatory activity	[Krakauer, Li & Young, 2001; Li et al., 2000; Wang et al., 2006]
Anti-bacterial activity	[Chung, Jin & Kim, 2003]
Anti-HIV activity	[Li et al., 2000; Wu et al., 2001]
Anti-SARS coronavirus activity	[Chen et al., 2004]
Anti-tumor activity	[Chan et al., 2000]
Free radical scavenging property	[Bochorakova et al., 2003; Gao et al., 2000; Wozniak, Lamer-Zarawska & Matkowski, 2004]
Antimutagenic property	[Wozniak, Lamer-Zarawska & Matkowski, 2004]
Induction of apoptosis	[Chan et al., 2000; Chang, Chen & Lu, 2002; Shieh et al., 2006]
Inhibition of proliferation, migration and differentiation	[Chang, Chen & Lu, 2002; Liu et al., 2003]
Inhibition of NF-kappa B activity	[Wan et al., 2008; Xue et al., 2006]
Prevention and treatment of periodontal disease	[Cai et al., 2008; Zhu, Li & Cao, 2007]
Antidepressant effect	[Zhu et al., 2006]
Antipyretic effect	[Tsai et al., 2006]
Anxiolytic-like effect	[Liao, Hung & Chen, 2003; Xu et al., 2006]
Ischaemic-like protective effect	[Liu et al., 2005]
Up-regulate TGF-beta 1 gene expression	[Chuang et al., 2005]
Inhibition of nitric oxide/cyclic GMP-mediated relaxation	[Huang et al., 2004]
Inhibition of intracellular Ca2+ elevation	[Kyo et al., 1998]

Table 2. Bioactivities of baicalein

Bioactivities	References
Antioxidant activity	[Chen et al., 2000; Chen et al., 2006; Zhao et al., 2000
Anti-inflammatory activity	[Lee et al., 2003; Suk et al., 2003; Woo et al., 2006]
Anti-bacterial activity	[Hahm et al., 2001]
Anti-viral activity	[Wu et al., 2001; Zofia et al., 2008]
Anti-cancer activity	[Bonham et al., 2005; Wang et al., 2004]
Free radical scavenging property	[Bochorakova et al., 2003; Shao et al., 2002; Wozniak, Lamer-Zarawska & Matkowski, 2004]
Inhibition of proliferation	[Chang et al., 2002; Lee et al., 2005; Lin et al., 2007]
Neuroprotective activity	[Cheng et al., 2008; Liu et al., 2006; Liu et al., 2007]
Cardioprotective effect	[Woo, Cheng & Waye, 2005]
Reduction of drug-induced adverse effect	[Sangeeta et al., 2007]
Anxiolytic-like effect	[Liao, Hun & Chen, 2003]
Against endotoxic effect	[Cheng et al., 2007]
Upregulatory activity in cell model	[Deng et al., 2008]
Proangiogenic activity	[Cho et al., 2008]
Suppression of iNOS expression	[Huang et al., 2007; Kim et al., 2001; Shen et al., 2002]
Antimutagenic effects	[Wozniak et al.,2004]
Neuroprotective effects	[Cho & Lee, 2004; Piao et al., 2004; Son et al., 2004]
Anticancer effects	[Chung et al., 2008; Lu et al., 2008; Zhang et al., 2008]
Antiinflammatory action	[Enomoto et al., 2007; Lim, 2004; Wang et al., 2007]
Anxiolytic effect	[Hui et al., 2002; Tai et al., 2005]
Anti-virus effect	[Blach-Olszewska et al., 2008; Guo et al., 2007; Ma et al., 2002]
Apoptosis inducement effect	[Baumann et al., 2008; Himeji et al., 2007; Lee et al., 2007]
Anticonvulsant effect	[Park et al., 2007]
Immunosuppressive action prevention	[Enomoto et al., 2007]
Inhibitory of ischemic brain injury	[Cho & Lee, 2004]
Protective effect on endotoxin-induced shock	[Van et al., 2001]

Table 3. Bioactivities of wogonin

Bioactivities	References
Suppression of iNOS expression	[Huang et al., 2007; Kim et al., 2001; Shen et al., 2002]
Antimutagenic effects	[Wozniak et al.,2004]
Neuroprotective effects	[Cho & Lee, 2004; Piao et al., 2004; Son et al., 2004]
Anticancer effects	[Chung et al., 2008; Lu et al., 2008; Zhang et al., 2008]
Antiinflammatory action	[Enomoto et al., 2007; Lim, 2004; Wang et al., 2007]
Anxiolytic effect	[Hui et al., 2002; Tai et al., 2005]
Anti-virus effect	[Blach-Olszewska et al., 2008; Guo et al., 2007; Ma et al., 2002]
Apoptosis inducement effect	[Baumann et al., 2008; Himeji et al., 2007; Lee et al., 2007]
Anticonvulsant effect	[Park et al., 2007]
Immunosuppressive action prevention	[Enomoto et al., 2007]
Inhibitory of ischemic brain injury	[Cho & Lee, 2004]
Protective effect on endotoxin-induced shock	[Van et al., 2001]

Table 4. Bioactivities of wogonin 7-0-glucuronide

Bioactivities	References
Antimutagenic property	[Wozniak et al., 2004]
Antiradical properties	[Bochorakova et al., 2003; Wozniak et al., 2003]
Antiinflammatory action	[Lim, 2003]

Table 5. Bioactivities of oroxylin A

Bioactivities	References
Neuroprotective effect	[Kim et al., 2006; Kim et al., 2007; Kim et al., 2008]
Anti-respiratory syncytial virus	[Ma et al., 2002]
Activate central nervous system	[Huen et al., 2003]

6.6. Other Bioactivities

The antioxidant and free radical scavenging, anti-inflammation, anticancer, antibacterium and antivirus properties of flavonoids of *S. baicalensis* Georgi have been discussed in Sections 6.1-6.5. In this section, the bioactivities of baicalin, baicalein, wogonin, wogonin 7-O-glucuronide and oroxylin A are summarized in Tables 1-5, respectively. As shown in Tables 1-5, the flavonoids of *S. baicalensis* Georgi have a variety of bioactivities.

7. Conclusion

Scutellaria baicalensis Georgi is one of the most widely used medicinal plants. It contains over 30 kinds of flavonoids, and baicalin, baicalein, wogonin, wogonin 7-O-glucuronide and oroxylin A are the main active components. The chemical structures, natural resources, biosyntheses, analytical methods and biological activities of the flavonoids have been summarized and discussed in this chapter. Tremendous progress has been achieved for the biosyntheses and biological activities of flavonoids in *S. baicalensis* Georgi. Biosyntheses of the flavonoids from *S. baicalensis* Georgi were carried out mostly using cells or organs culture system, and the strategies used for improving the flavonoids production efficiency included media optimization, biotransformation, elicitation, Agrobacterium transformation and scale-up. Most of the biological activities, such as antioxidant and free radical scavenging, anti-inflammation, anticancer, antibacterium and antivirus, are closely related to the clinical applications of *S. baicalensis* Georgi for anti-inflammation, anticancer, reducing the total cholesterol level, treating bacterial and viral infections of the respiratory and the gastrointestinal tracts. In the future, bioactive components at highly purity should be used instead of crude extracts in medicinal preparations. In order to explore more effective herbal products based on *S. baicalensis* Georgi, more widely pharmacological studies should be carried out to determine new pharmacodynamic effects, such as anti-SARS coronavirus. In addition, more attention should be paid to minor flavonoids in *S. baicalensis* Georgi because special pharmacodynamic effects may be found from minor flavonoids. The structural diversity and the pronounced biological activities encountered in the flavonoids of *S. baicalensis* indicate that this class of compounds is worthy of further studies that may lead to new drug discovery.

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Chapter 5

Management of Diabetes with Diet and Plant–Derived Drugs

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Abstract

Diabetes is a metabolic syndrome resulting from low levels of insulin. Common symptoms are hyperglycemia, polyuria, polydipsia, blurred vision, lethargy and weight loss. The increasing worldwide incidence of diabetes mellitus in adults constitutes a major global public health burden. The World Health Organization (WHO) estimates that currently more than 180 million people worldwide have diabetes. This number is likely to double by 2030 when it is predicted that India, China and the United States will have the largest number of people with diabetes.

Plants have been the main source of medicines since ancient times. Despite tremendous advances in medicinal chemistry, synthetic drugs have not provided cures to many diseases due to their adverse side effects or diminution in response after prolonged use. Plants are the richest source of natural compounds and continue to provide new chemical entities for the development of drugs against various diseases like cancer, diabetes, inflammation, hypertension and neurodegeneration. As such, there is renewed interest in traditional medicines with the belief that plant-derived drugs are generally less toxic and safer than synthetic drugs. With respect to diabetes, numerous studies have indicated that plant-derived chemicals may be useful in the therapeutic treatment of diabetes. However, before the development of therapeutic insulin, diet was (and still is) the main method of treatment and modern treatment focuses on a combination of drugs and diet. Dietary measures included the use of traditional medicines mainly derived from plants. While drugs will continue to be an important part of diabetes therapy, the mass of evidence available in the literature regarding the medicinal properties of vegetables, fruits and other herbs, suggests that diet (including herbal medicines) should not be ignored or neglected.

This review will focus on recent examples of traditional medicines and foods that have been validated by scientific evaluation as having promising activity for the

prevention and/or treatment of diabetes. Intriguing questions that await further elucidation include how plants, plant-derived molecules and diet can be used in the future to complement current treatment strategies for diabetes.

Introduction

Diabetes mellitus is a metabolic syndrome which results from low levels of insulin when β cells of the pancreas are not able to secrete sufficient insulin. The symptoms of diabetes are hyperglycemia (high blood glucose), polyuria (increase in urine production), polydipsia (increased thirst), blurred vision, lethargy and weight loss. The increasing worldwide incidence of diabetes mellitus in adults constitutes a global public health burden. It is predicted that by 2030, India, China and United States will have largest number of people with diabetes (1, 2). The World Health Organization (WHO) estimates that more than 180 million people worldwide currently have diabetes and this figure is likely to more than double by 2030. In 2005, an estimated 1.1 million people died from diabetes and almost 80% of diabetes deaths occurred in low and middle-income countries. Almost half of diabetes deaths occur in people under the age of 70 years; 55% of diabetes deaths are in women and the WHO projects that diabetes death will increase by more than 50% in the next 10 years without urgent action. Most notably, diabetes deaths are projected to increase by over 80% in upper-middle income countries between 2006 and 2015 (3).

Over time, diabetes can damage the heart, blood vessels, eyes, kidneys, and nerves. The major complications related to diabetes are *diabetic retinopathy*, a cause of blindness which results from long-term accumulated damage to the small blood vessels in the retina, and *diabetic neuropathy,* the destruction of nerves as a result of diabetes with common symptoms of tingling, pain, numbness, or weakness in the feet and hands. Combined with reduced blood flow, neuropathy in the feet increases the chance of foot ulcers and eventual limb amputation. Diabetes is among the leading causes of kidney failure and increases the risk of heart disease and stroke. Ten to twenty percent of people with diabetes die of kidney failure and fifty percent of people with diabetes die of cardiovascular disease (primarily heart disease and stroke). The overall risk of dying among people with diabetes is at least double the risk of their peers without diabetes (3).

[153] Diabetes is categorized into: Type I – Insulin Dependent Diabetes Mellitus (IDDM) – which is an autoimmune destruction of pancreatic β cells; Type II – Non-Insulin Dependent Diabetes Mellitus (NIDDM) – which is characterized by insulin resistance in target tissues; and gestational diabetes which occurs during pregnancy.

In Type I diabetes, there is loss of insulin secreting β cells of the Islets of Langerhans in the pancreas which causes deficiency of insulin. The main cause of β-cell loss is autoimmune attack of T-cells. The principal treatment is replacement of insulin.

Type II diabetes is caused by reduced insulin sensitivity due to increased glucose levels in the blood. Hyperglycemia can be rectified by medications that improve insulin sensitivity or decrease glucose production by the liver. This condition can be treated by increasing physical activity, decreasing carbohydrate intake, selection of proper diet, modification of life-style and losing weight.

Gestational diabetes, although temporary, increases the risk of developing Type II diabetes later in life. Although insulin injections are sometimes necessary, this type of diabetes is also commonly treated by life style changes such as moderate physical activity and diet.

The principal clinical features of diabetes mellitus were described by Hindu scholars as long ago as about 1500 BC as a condition featuring polydipsia, polyuria and the production of urine which was sweet enough to attract flies and ants (4). The current focus of drug discovery research in diabetes includes exploration of alternative medicines, discovery of new synthetic antidiabetic agents as well as isolation of active compounds from plants which have been the source of traditional herbal medicines and have been documented and described for their antidiabetic properties in ancient texts like Ayurveda. The WHO has recommended that alternative medicines should be investigated and explored for discovery of new drugs for the treatment of diabetes mellitus (5).

Current Therapies for Diabetes

Currently available therapies for diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides, α-glucosidase inhibitors, α-amylase inhibitors and glinides, which are used as monotherapy or in combination to achieve better glycemic regulation. The medications available in the market, and their side effects, are as follows and are summarized in Figure 1:

- **Metformin,** is the only biguanide available to most of the world and its major effect is to decrease hepatic glucose output and lower fasting glycemia. It is generally well tolerated, with the most common adverse effects being gastrointestinal.
- **Sulfonylureas** lower glycemia by increasing insulin secretion. The major adverse effect is hypoglycemia, while weight gain is also a common concern.
- **Glinides,** like the sulfonylureas, also stimulate insulin secretion but bind to a different site within the sulfonylurea receptor and have a shorter half-life than the sulfonylureas and therefore must be administered more frequently. The glinides have a similar risk for weight gain as the sulfonylureas, but hypoglycemia may be less frequent (nateglinide) than with some sulfonylureas.
- **Enzyme inhibitors** lower the rate of digestion of polysaccharides in the proximal small intestine, primarily lowering postprandial glucose levels without causing hypoglycemia. Since carbohydrates are absorbed more distally, malabsorption and weight loss are ameliorated, however, increased delivery of carbohydrate to the colon commonly results in gas production and gastrointestinal symptoms.
- **Thiazolidinediones (TZD)** or **glitazones** are peroxisome proliferator–activated receptor γ modulators which increase the sensitivity of muscle, fat, and liver to endogenous and exogenous insulin (“insulin sensitizers”). The most common adverse effects with TZDs are weight gain and fluid retention.

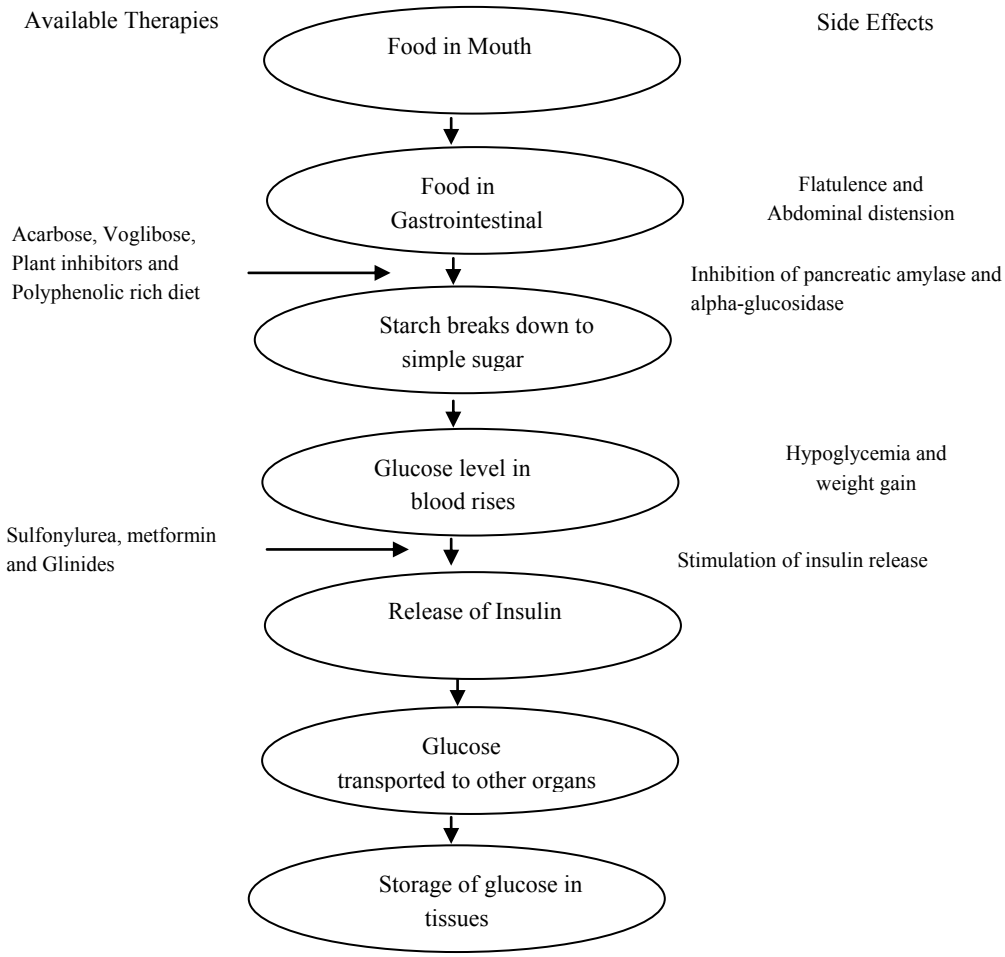
Available Therapies
Side Effects
Food in Mouth
Food in Gastrointestinal
Flatulence and Abdominal distension
Acarbose, Voglibose, Plant inhibitors and Polyphenolic rich diet
Inhibition of pancreatic amylase and alpha-glucosidase
Starch breaks down to simple sugar
Glucose level in blood rises
Hypoglycemia and weight gain
Sulfonylurea, metformin and Glinides
Stimulation of insulin release
Release of Insulin
Glucose transported to other organs
Storage of glucose in tissues

Figure 1. Digestion of food and mechanisms of currently available therapies, and their side effects, for diabetes.

- **Insulin** is the oldest among the currently available medications. Initially developed to treat Type I diabetic patients for whom it is life saving. Insulin is the most effective of the diabetes medications in lowering glycemia. Insulin therapy has beneficial effects on triglyceride and HDL cholesterol levels but is also associated with weight gain (6).

It is clearly seen that enzyme inhibitors such as diet, vegetables, and fruits rich in polyphenolic compounds have bearable side effects as compared to other oral hypoglycemic agents.

Although, oral hypoglycemic agents and insulin play important roles in the treatment of diabetes by controlling hyperglycemia, these have serious side effects which may cause other diabetic complications and most of the medicines available in the market are associated with the adverse consequences of hypoglycemia or weight gain (7). Thus, treatment of diabetes without any side effects is still a challenge (8). When selecting an appropriate therapy for Type II diabetes, then, factors such as other co-existing medical conditions (high blood

pressure and elevated cholesterol), adverse effects of that therapy, contraindications to therapy, issues which may affect compliance (timing of medication, frequency of dosing) and cost to the patient and the healthcare system should be considered alongside the magnitude of change in blood sugar control that each medication will provide. Moreover, the relatively complication-free option of diet and life style change should be considered.

Management of Diabetes with Traditional Medicinal Plants and Dietary Control

An important research area is the discovery and development of more effective and safer antidiabetic agents. In this context, medicinal plants and diet continue to play an important role in the treatment of diabetes, particularly in developing countries where most people have limited resources and do not have access to modern treatment (5). A recent survey of the frequency of use of complementary and alternative medicine (CAM) in diabetes patients found that most of the patients using CAM are better educated, born in cities, live in large families and were suffering from diabetes for longer duration. This included herbal preparations (garden thyme, pomegranate syrup, stinging nettle, dog-rose, chervil, cinnamon, and bitter almond), acupuncture and meditation. Further, it was reported that more than half of the subjects who were using CAM experienced beneficial effects (9).

Many plants and their active chemical compounds have demonstrated activity in the treatment of Type II diabetes and various other disorders. According to ethno botanical information, more than 800 plants are used as traditional remedies in one or other form for the treatment of diabetes (10). Many different moieties, chemical groups and chemical constituents with therapeutic efficacy have been isolated and purified from plants which were traditionally used to treat disease. One should note that metformin, the single most prescribed agent for the treatment of diabetes, originated from herbal medicine (11, 12) and was derived from galegine. Experimental and clinical evaluations of galegine, isolated from *Galega officinalis* provided the pharmacological and chemical basis for the subsequent discovery of metformin (11, 13). 1- Deoxynojirimycin (DNJ), a potent α-glucosidase inhibitor which helps in prevention of diabetes, was isolated from the water extract of leaves of mulberry trees (*Morus alba* L.) (14).

Diet has long been the keystone in the treatment of diabetes and various other diseases. The Ebers Papyrus prescribed in 1550 BC that a diet rich in wheatgerm and ochra has glucose-lowering efficacy (12). Diet and lifestyle play an important role in the management of several diseases, including diabetes. Before the introduction of the therapeutic use of insulin, diet was the main form of treatment and dietary measures included the use of traditional medicines mainly derived from plants (15).

The ancient Indian medical system of Ayurveda, which is based on scientific principles, has also described diabetes under the name madhumeha, stating it to be mainly influenced by dietary factors such as excessive eating of sugary, acidic or salty food; certain non-vegetarian foodstuffs; and lifestyle factors such as lack of exercise, overindulgence in sleep, sedentary habits, lack of cleanliness and “suppression of natural urges”. Current studies have confirmed

that there is increased risk of developing Type II diabetes from lack of exercise and sedentary lifestyle (16).

Many studies have confirmed the benefits of medicinal plants with hypoglycemic effects in the management of diabetes mellitus. The effects of these plants may delay the development of diabetic complications and correct metabolic abnormalities. Moreover, during the past few years, some of the new bioactive drugs isolated from plants showed antidiabetic activity with more efficacy than oral hypoglycemic agents used in clinical therapy (17).

The folk medicines used for the treatment and prevention of diabetes include garlic, onion, ginseng, bitter melon, fenugreek, *Gymnema sylvestre, Pterocarpus marsupium* and other plants containing flavonoid compounds, bilberry, aloe vera, and holly. The active ingredients derived from plants used for antidiabetic preparations have been identified, and potentially beneficial effects on the rate of food ingestion, glucose transport, potentiation of insulin release, inhibition of insulin clearance, insulin-mimetic effects, reduced gluconeogenesis, and β-cell protection have been attributed to these agents (18). Some plants, such as *G. sylvestre*, *M. charantia* and *P. marsupium*, may also help in regeneration of β cells in the pancreas, which is an important discovery because none of the conventional oral hypoglycemic agents shows this action (5).

Dietary management of diabetes includes consumption of food, spices, fruits, vegetables, traditional medicines and herbs. The diet should provide adequate amounts of vitamins, minerals, carbohydrates, fats and proteins. Diets which enhance glycemic control are high in fibre, low to moderate in fats and moderate in biological value proteins like legumes, beans, vegetables, soy and other plant based proteins which our body can digest, absorb and utilize easily. The decrease of calorie intake in diabetic patients helps in weight loss. Diets rich in fibre and containing 60% carbohydrates improve blood sugar and lipid levels. Thus, dietary modification is the first line of therapy for diabetic patients. Dietary strategies normalize blood glucose and lipoprotein levels to reduce morbidity and mortality caused by disturbance in carbohydrates and lipoprotein metabolism in diabetes mellitus. These goals can be achieved by considering the quantity and quality of diets according to the clinical conditions of an individual (19).

Some examples of dietary management of diabetes which have been evaluated scientifically are described below.

A considerable number of human and animal experiments have been carried out to evaluate the efficacy of common spices and natural food adjuncts for several physiological effects such as antidiabetic, digestive stimulant, cholesterol lowering, anti-carcinogenic, anti-inflammatory, anti-oxidant and anti-lithogenic potential. Several common spices such as fenugreek (*Trigonella foenumgraecum*) were studied on diabetic and normal rats, mice, rabbits and dogs which was also confirmed by human clinical trials that fenugreek possess beneficial hypoglycemic potential. Garlic (*Allium sativum*), onion (*Allium cepa*), cumin (*Cuminum cyminum*), turmeric (*Curcuma longa*) are some other spices with beneficial anti-diabetic properties (based on animal studies). Experimental data indicated that dosages of 25-50 grams of fenugreek seeds, 5-6 garlic cloves, 1 onion bulb, and 1 gram of turmeric powder incorporated into the daily diet of diabetics were effective as a support therapy in the prevention and management of diabetes and related complications like hypertension and

obesity. The mechanisms of action are recognized as stimulation of the pancreas to secrete insulin, interference with dietary glucose absorption and insulin sparing action of bioactive compounds. Ginger (*Zingiber officinale*), curry leaf (*Murraya koenigii*), mustard (*Brassica nigra*) and coriander (*Coriandrum sativum*) also improved glucose tolerance in experimental diabetic animals (20).

Apart from serving as flavouring agents, spices can also be used in the management of certain metabolic disorders such as diabetes. *Rhus coriaria* L., also called sumac, and *Bunium persicum* Boiss, also known as black Persian cumin, are two spices used as a condiment, particularly in Iran and Afghanistan. The methanolic, ethyl acetate and n-hexane extracts of both the spices have been studied for their ability to inhibit the enzyme α-amylase. The ethyl acetate extract of *Rhus coriaria* fruits showed significant α-amylase inhibitory activity and thus has the potential to be used in the management of diabetes (21).

Various samples of fruit-enriched yoghurts have been tested for diabetes and hypertension management. Dairy and soy yoghurt enriched with strawberry, blueberry and peach were screened, *in vitro*, for total phenolic content, antioxidant activity, α-glucosidase inhibition, α-amylase inhibition and the angiotensin converting enzyme-I (ACE-I) inhibition. Soy yoghurt enriched with blueberry showed the highest antioxidant activity, phenolic content, α-glucosidase inhibition and α-amylase inhibition. The results indicated that enrichment of yoghurts with fruit phytochemicals like blueberries showed high health functional value in terms of Type II diabetes management. Soy yoghurt, enriched with blueberries, appeared to be the best food system in the management of diabetes and its long term complications (22). Cheese, another beneficial dairy product, has been evaluated against the key enzymes linked to Type II diabetes and hypertension. Three different types of cheese – cheddar, feta and Roquefort have been screened to determine their potential to inhibit α-glucosidase, α-amylase and ACE-I. All samples of cheese showed very high ACE-I inhibition, while cranberry-enriched cheeses had the highest activity for α–glucosidase and α-amylase inhibition. Therefore, cheeses enriched with cranberries have promising anti-diabetic potential such that enrichment with herbs and fruit phytochemicals can result in the enhanced health functional value of cheese in relation to Type II diabetes management (23).

The aqueous extracts of some American foods (fresh green pepper, string beans, baby spinach, broccoli sprouts, red pepper, fresh carrot, romaine lettuce, red grape, tomato and basil leaves, graham cracker, chips ahoy cookies and wheat thins crackers) and Asian foods (powdered Asian spices fenugreek, mustard, ginger, cinnamon, turmeric, fennel powder, cardamom powder, fresh eggplant, coccinia, bittergourd, small brinjal, ginger, mustard and fresh carrot) were screened using *in vitro* enzymatic assays. Overall, Asian foods were found to be more active than the American foods and possessed higher antioxidant activity and α-amylase inhibition. Red grape, green pepper, broccoli sprouts, fresh carrot, ginger, coccinia, mustard and cinnamon extracts had the strongest anti-α-amylase activities. Wheat thin crackers, red grape, broccoli sprouts, green pepper, cinnamon, fenugreek, fennel powder and ginger had minor α-glucosidase inhibitory activity. However, ginger extract was found to possess significant anti-ACE activity which shows that ginger may also have a strong potential as an antihypertensive agent. It was suggested that antioxidant activity was associated with amylase inhibition, protein content seemed to be inversely associated with the amylase inhibition and protein–phenolic and/or phenolic–phenolic synergies may be involved

in the food extract enzyme-inhibition mechanism. The results from these experiments showed that common vegetables and spices contained significant antidiabetic activity *in vitro*, as well as anti-ACE activity, and suggested that dietary modification to include these types of foods along with balancing carbohydrate intake throughout the day may represent a promising strategy to help control postprandial hyperglycemia through modulation of carbohydrate absorption. Dietary α-amylase and α-glucosidase inhibitors from common foods are potentially safer, therefore, may be a preferred alternative for the reduction of carbohydrate absorption and control of blood glucose (24).

The inhibitory effects of polyphenol components of berries on various digestive enzymes were studied and it was found that anthocyanins inhibit *α*-glucosidase and reduce blood glucose levels after ingestion of meals rich in starch, and they may therefore control hyperglycemia. Ellagitannins, which are present in berries, inhibit *α*-amylase activity. Raspberries and strawberries contain high amounts of ellagitannins and anthocyanins. Berry polyphenols like flavonols, anthocyanidins, ellagitannins and proanthocyanidins can inhibit protease enzyme which could, in turn, affect protein digestion in the gastrointestinal tract. Proanthocyanidins can inhibit gastrointestinal lipase activity which helps in the control of obesity by reducing fat digestion. Polyphenol components in berries, fruits and other vegetables provide health benefits by inhibition of these digestive enzymes thus providing an alternative to pharmaceutical and nutraceutical treatment for non-insulin dependent diabetes and obesity (25).

The National Diabetes Education Program of the National Institutes of Health (NIH) recommends that eggplant should be included in the diet for the management of Type II diabetes. The phenolic-enriched antioxidant activity and α-glucosidase inhibitory potential might help to reduce hyperglycemia-induced pathogenesis. This was tested experimentally *in vitro* by extracting four varieties (Purple, White, Graffiti, Italian) of fresh and well-ripened eggplant (*Solanum melongena)*, with water and screened for activity using α-amylase, α-glucosidase and ACE-I inhibition, DPPH and total phenolic assays. The results indicated that phenolic-enriched extracts had high α-glucosidase inhibitory activity, moderate antioxidant activity and moderate to high ACE-I inhibitory activity. Eggplant may therefore control glucose absorption and decrease the risk of related hypertension because of its high fibre, phenolic compounds and low soluble carbohydrate content. Inhibition of these enzymes provide strong biochemical basis for management of Type II diabetes by controlling glucose absorption and associated hypertension. The phenolic antioxidant-enriched dietary strategy also has the potential to reduce cellular oxidation stress which is also related to diabetes (26).

In an *in vitro* epididymal fat cell assay, tea has been shown to increase insulin activity. Black, green, oolong and herbal teas all increased insulin activity and the insulin potentiating activity of green and oolong teas was considered to be due to epigallocatechin gallate. The other compounds responsible for enhancing insulin activities are epicatechin gallate, tannins and theaflavins (27). These data were supported by *in vitro* enzyme inhibition analysis of other phenolic phytochemicals including the four types of tea (green tea, oolong tea, black tea and white tea) and several varieties of red and white wine. The aqueous extract of black tea showed the highest α-glucosidase inhibition followed by white and oolong tea. Red wines had high α-glucosidase inhibition compared to white wine and the inhibitory activity was correlated to phenolic content, antioxidant activity and phenolic profile of the extracts. These

extracts showed less α-amylase inhibition which indicates the potential to overcome the side effects of undigested starch, and thus may have benefits for the management of hyperglycemia (28). Routine consumption of green tea has been reported as showing beneficial effects on various metabolic disorders such as Type II diabetes, obesity and cardiovascular risks because of its catechin (specifically EGCG (-)-epigallocatechin-3-gallate) content in various *in vitro* and animal studies (29).

Varieties of pumpkin (*Cucurbita pepo*), maize (*Zea mays*) and beans (*Glycine max*, *Vigna angularis*, *Canavalia* spp., *Cicer arietinum*, and *Canavalia ensiformis*) have been screened using *in vitro* enzyme (α-glucosidase, α-amylase and ACE-I) inhibition assays. Round orange and spotted orange green pumpkin extracts had the highest content of total phenolics and moderate antioxidant activity, the highest potential for glucosidase and ACE-I inhibition and may thus help in reducing hyperglycemia and associated complications linked to cellular oxidation stress and hypertension. Selected types of pumpkin, beans and maize varieties have moderate phenolic content with moderate free radical scavenging linked antioxidant activity and thus may be of value in reducing hyperglycemia-induced microvascular complications (30).

Aqueous extracts of nine types of pepper, *Capsicum annum*, (green, red, orange, yellow, cubanelle, red sweet, yellow sweet, long hot and jalapeno) were investigated for inhibitory activities against α-glucosidase, α-amylase and ACE-I. Green, red sweet, long hot and yellow sweet had high inhibitory activity against α-glucosidase from both rat intestine and yeast; red sweet possessed highest α-amylase activity and yellow pepper had the highest ACE-I inhibitory activity followed by cubanelle, red and red sweet. Some peppers showed high α-glucosidase with low α-amylase activity which could be a good dietary strategy to control glucose absorption without the side effects of undigested starch. This study indicated that peppers are rich in phenolic phytochemicals and have high free radical scavenging-linked antioxidant activity. These foods have the potential to reduce hyperglycemia-induced vascular complications and tissue damage resulting from oxidation and help reduce hyperglycemia and related long term complications of diabetes (e.g. hypertension) (31). Dried cranberry powder, dried oregano, and rosemary powders were screened using the same enzyme assays mentioned above. Water extracts of pure dried oregano exhibited the greatest α-glucosidase and α-amylase inhibition, water extracts of oregano had the greatest DPPH radical inhibition activity and pure cranberry had the greatest ACE-I inhibitory activity (32).

Legumes, including soybeans chickpeas, lentils, kidney beans, cannellini beans, soybeans, berlotti beans, baked beans and peanuts, reduce the risk of developing Type II diabetes as they are low in fat, high in fibre, are a good source of protein and have low glycemic index. Animal studies of obesity and diabetes showed soybeans reduced serum insulin and insulin resistance, while a study of middle aged Chinese women has also shown that consumption of legumes, in particular soybeans, was inversely associated with the risk of Type II diabetes (33). Alpha-amylase inhibitor (α-AI) has been isolated and purified from kidney beans (*Phaseolus vulgaris* L. cv Tendergreen). Two isoforms, α-AI1 and α-AI1', of 43 kDa have been isolated with a difference in their isoelectric point and neutral sugar content. The major isoform, α-AI1 inhibited human and porcine pancreatic α-amylase (PPA) but not bacterial or fungal α-amylase enzymes (34). Douchi, a fermented soybean Chinese food, has been screened for α-glucosidase inhibition and found to have high activity. Douchi sourced

from three different parts and fermented with three different fungal strains was also shown to possess significant α-glucosidase inhibitory activity. Douchi fermentatied with *A. oryzae* had strong inhibition as compared to the same food fermented with other fungi like *A. elegans* and *R. arrhizus* (35). Genistein, an isoflavone isolated from soybeans is a potent α-glucosidase inhibitor (36).

The anti-diabetic potential of ten plants, agrimony (*Agrimony eupatoria*), coriander (*Coriandrum sativum*), eucalyptus (*Eucalyptus globulus*), juniper (*Juniperus communis*), Lucerne (*Medicago sativa*), avocado (*Persea americana*), elder (*Sambucus nigra*), nettle (*Urtica diocia*), mushroom (*Agaricus campestris*) and mistletoe (*Viscum album*) were evaluated by an *in vitro* dialysis model of glucose movement. The glucose movement was decreased by more than 50% by agrimony and avocado. Mushroom, coriander, eucalyptus, juniper, lucerne, and mistletoe were less effective. Nettle and elder extracts did not significantly decrease glucose diffusion. The effects of agrimony, avocado, coriander and mushroom extracts were found to be concentration dependent. It was concluded that agrimony and avocado have the ability to inhibit glucose diffusion using an *in vitro* model of glucose absorption and represented potential dietary supplements that may be useful for allowing flexibility in meal planning for management of Type II diabetes (37). In an *in vivo* study, the aqueous and methanolic leaf extracts of avocado resulted in a reduction in plasma glucose level, total cholesterol and LDL-cholesterol levels in albino rats (38). The methanolic extract of the flowering part of pomegranate (*Punica granatum* Linn.) was evaluated by *in vivo* and *in vitro* diabetes assays. The extract was shown to decrease plasma glucose levels and possess potent inhibitory activity against α-glucosidase. It was suggested that it improved postprandial hyperglycemia during treatment of Type II diabetes and obesity (39). The extracts of strawberries (*Fragaria ananasia*) and raspberries (*Rubus idaeus* L. variety Glen Ample) significantly inhibited salivary as well as pancreatic α-amylase enzyme. Blueberries (*Vaccinium corymbosum* L. variety Berkley), blackcurrant (*Ribes nigrum* L. variety Ben Lomond), red cabbage, red wine, red grape and green teas were also shown to be effective and it was found that the activities were due to soluble tannins in these fruit extracts. Blueberries and blackcurrants were shown to be more active against α-glucosidase and the activity was based on their anthocyanin content. The inhibitory activity of anthocyanins and tannins was proved by removing the anthocyanin and tannin fractions from the above samples, and it was reported that tannins were related to amylase inhibition while anthocyanins were responsible for glucosidase inhibition (40).

In another study, a new natural α-glucosidase inhibitor from red wine vinegar (made by the fermentation of storage root paste of purple fleshed sweet potato, *Ipomea batata*) was identified as caffeoylsophorose. The compound was tested against α-glucosidase and studied in Sprague Dawley rats and the experiments demonstrated that caffeoylsophorose suppressed the increased postprandial blood glucose level achieved by inhibition of maltase (41).

Clonal herbs of family Lamiaceae were evaluated for the management of diabetes and hypertension. Water extracts of clonal lines of rosemary *Rosmarinus officinalis,* clones (Rosemary LA, Rosemary RoK-2 and Rosemary Ro-6), lemon balm (*Melissa officinalis*), sage (*Salvia officinalis*), chocolate mint (*Mentha piperata*) and oregano (*Origanum vulgare*, clone Oregano Go-19-2) were screened using enzymatic inhibition assays of α-glucosidase, α-amylase and ACE-I. Oregano showed the greatest α-glucosidase inhibition activity,

followed by chocolate mint and lemon balm. Clonal lines of rosemary also showed significant α-glucosidase inhibition. ACE-I inhibition activity was greatest in rosemary, rosemary LA followed by lemon balm and oregano (42). Other nutraceutical compounds which reduce the risk of diabetes are found in diets rich in fibres, legumes, coffee (chlorogenic acid), barley malt, biotin, magnesium, chromium picolinate, calcium/vitamin D, bitter melon, cinnamon extracts (43). Hot water extracts of coffee seeds showed significant inhibition against both the enzymes α-glucosidase and α-amylase and reduced postprandial hyperglycemia as assessed by *in vivo* assays on Wistar rats for Oral Saccharinity tolerance test (OST) (44).

Consumption of other foodstuffs which are digested at slower rates is a good strategy to manage diabetes and its related complications of obesity and hypertension. Grains which are rich in β-glucans, such as Prowashonupana (a cultivar of barley that is less digestible than regular barley) are good for diabetic patients. Both barley varieties have been studied for their digestion and absorption and it was found that absorption of Prowashonupana was lower compared to barley (45). Whole wheat seeds, partially decorticated wheat (belila), fenugreek seed powder, fenugreek germinated seeds, lupine, chickpeas and composite biscuits of whole wheat/fenugreek and whole wheat/chickpea also showed good effect in diabetes patients. It has been reported that daily consumption of whole grain foods and legumes in many forms improves glucose tolerance and serum insulin levels in diabetic patients (46). The phenolic compounds of finger millet or ragi (*Eleusine coracana* L.) from the seed coat have been screened against α-glucosidase and pancreatic amylase and found to exhibit strong inhibition against both enzymes (47).

Bitter gourd or bitter melon (*Momordica charantia* L.) is consumed as a vegetable and herbal medicine in various parts of the world is considered to prevent and help in the management of diabetes and its related complications. It has been proved by a cell culture and glucose uptake assay that the hypoglycemic potential of bitter gourd was due to activation of AMP-Activated protein kinase (48). Leaves of *Tamarindus indicus* showed 90% inhibition of α-amylase (49). The by-products of the processing of pineapples, *Ananas cosmosus*, (i.e. remaining pulp, peels and skin) are rich in phenolic compounds, soluble sugars and high in fibre. After being dried, ground and mixed with organic soy bean flour in a ratio of 1:1 and 9:1 and bio-processed with *Rhizopus oligosporus* for 12 days, the 9:1 mixture showed the highest level of α-amylase inhibition after 2 days of *R. oligosporous* growth (50).

Other fruits and vegetables which have been reported as helping to decrease hyperglycemia are the rind of bitter cucumber (*Citrullus colocynthis* Schard), roots of cabbage (*Anthocleista voglii*), fruits of *Eugenia jambolana* Lam. *Syzigium cumini* Skeels (Jamun), seeds of Malabar kola (*Garcinia kola*), leaf extract of mango (*Mangifera indica*), flowers and fruits of banana (*Musa sapientum* Kuntze), leaves of olive (*Olea europea* L.), seeds of pigeon pea (*Cajanus cajan* Millsp.), leaves of mulberry (*Morus alba* L.), *Eriobotrya japonica* Lindl. (loquat), leaves of jackfruit (*Atrocarpus heterophyllus* Lam.), leaves of black tea (*Camellia sinensis* L.), roots of ginger (*Zingiber officinalis*), fruits of custard apple (*Annona squamosa*), husk of isphagula (*Plantago ovate*), bitter gourd (*Momordica Charantia*), Ivy gourd (*Coccinia indica*), leaves of mustard (*Brassica juncea*), cinnamon (*Cinnamomi cassia*), tubers of onion (*Allium cepa* L.), *Beta vulgaris* var. Cicla L., *Aegle marmelose.* In addition, other well-known plants with this activity are *Aloe barbedensis,*

Ocimum album, Achyranthes aspera, Withania somnifera, Salacia oblonga, Equisetum myriochaetum, Salacia oblonga Wall, *Swertia chiraita, Swertia japonica, Aralia cachemirica* Decne., *Cryptolepis Sanguinolenta, Ocimum sanctum* Linn, *Stevia rebaudiana* Bertoni, *Cantharanthus roseus, Azadirachta indica, Mucuna pruriens, Eruka sativa, Opuntia steptacantha, Lantana camara, Agrimony eupatoria* L., *Eucalyptus globules* Labill, *Semecarpus anacardium* Linn., *Chamaemelum nobile, Salvia officinalis, Coscinium fenestratum, Pterocarpus marsupium, Asparagus adscendens, Selaginella tamariscina* Beauv, *Nelumbo nucifera, Phyllanthus amarus, Tinospora cardifolia, Acanthopanax senticosus, Silybum marianum, Panax ginseng, Aesculus hippocastanum* L., *Kochia scoparia, Salvia lavandifolia* Vahl., *Butea monosperma, Gymnema sylvestre, Acrocomia mexicana, Pandanus odorus* and *Salicornia herbacea* L. (19). An extract of pine bark and needle showed inhibition against salivary α-amylase and yeast α-glucosidase enzymes and significantly reduced postprandial glucose level (51). The astringent extract of chest nut skin (52), extract of *Pycnanthus angolensis* fruits (53), ethanolic extract of *Butea monosperma* (54), leaf extract of kiwi fruit (55), seed kernel of *Syzigium cumini* (56), leaves of guava, *Psidium guajava* Linn. (57) have all shown good hypoglycemic potential.

Pytochemicals with Anti-Diabetic Activities

A number of bioactive compounds have been isolated from plants which are potent α-glucosidase and/or α-amylase inhibitors and show good antidiabetic properties. The main phytochemicals with reported anti-diabetic activities were flavonoids, polyphenolic compounds, tannins, glycosides, alkaloids and terpenoids. The active phytochemicals were isolated, purified and scientifically validated for anti-diabetic action by *in vitro* or *in vivo* experiments.

Flavonoids and Polyphenolic compounds: Luteolin isolated from *Lonicera japonica*, amentoflavone isolated from the leaves of *Ginkgo biloba*, luteolin-7-O-glucoside isolated from *Salix gracilistyla* and daidzein isolated from soybeans are all natural flavanoids which showed strong inhibitory activity against α-glucosidase and α-amylase, with luteolin exhibiting greater activity than acarbose (58). Similarly, hydnocarpin, luteolin and isohydnocarpin isolated from acetone extracts of seed hulls of *Hydnocarpus wightiana* Blume were screened against yeast α-glucosidase and it was found that luteolin showed the strongest inhibitory activity; isohydnocarpin was also a potent inhibitor while hydnocarpin was a mild inhibitor (59). Quercetin 3-O-β-D-xylopyranosyl (1″ ′→ 2″′)-β-D-galactopyranoside and (-)-lyoniresinol 3-O-β-D-glucopyranoside isolated from the leaves of *Alstonia scholaris*, also called as Devil tree, is a traditional Thai medicinal plant. Quercetin 3-O-β-D-xylopyranosyl (1″ ′→ 2″′)-β-D-galactopyranoside was found to possess maltase inhibitory activity and (-)-lyoniresinol 3-O-β-D-glucopyranoside showed significant inhibition against both the sucrase and maltase activities of α-glucosidase (60).

Crude 50% methanolic extracts of rhizomes of *Berginia ciliata*, a Nepalese medicinal plant used to treat several diseases, showed significant inhibitory activity against rat intestinal α-glucosidase and porcine pancreatic α-amylase. This extract was fractionated for the

isolation of novel active compounds which were further screened for activity against the same enzymes. (-)-3-O-galloylepicatechin and (-)-3-O-galloylcatechin were isolated as potent antidiabetic compounds which showed dose-dependent enzyme inhibition (61). Chebulanin, chebulagic acid and chebulinic acid isolated from *Terminalia chebula* have been shown to possess potent inhibitory activity against α-glucosidase (62). In another study, chebulagic acid from *Terminalia chebula* also showed good anti-diabetic activity (63).

Tussilago farfara L. is a common plant in China used as a folk medicine. The aqueous methanolic extract of flower buds of this plant and the isolated compounds 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and rutin showed good maltase inhibitory activity of rat intestinal α-glucosidase, and thus may help in reduction of postprandial hyperglycemia (64).

In another study it was suggested that unripe banana (*Musa paradisiaca* L.) flour be used to make pasta or spaghetti to increase the undigestible carbohydrates and increase the antioxidant content. Green unripe banana is rich in proanthocyanidins, polyphenolic compounds and vitamins and thus possesses significant antioxidant activity. Moreover, the flour is considered to be of low glycemic index food, high in resistant starch and non-starch polysaccharides and possess slow carbohydrate absorption which could be a good strategy to prevent diabetes (65). An aqueous extract of unripe plantain (*Musa paradisiaca*) was shown to possess hypoglycemic activity, as it reduced glucose levels in normal and alloxan-induced diabetic rats (66).

MC2-1-5, a water soluble peptide purified from *Momordica charantia* L.Var. Abbreviata Ser. showed significant hypoglycemic potential. When studied in alloxan-induced diabetic mice, it significantly reduced the blood glucose level (67). The hot water extract of chamomile, *Matricaria chamomilla* L., and the isolated compounds esculetin and quercetin, help in prevention of hyperglycemia and the reduction of diabetic complications in diabetes patients. It was suggested that daily consumption of chamomile tea can prevent hyperglycemia and diabetic complications (68).

Six groups of flavonoids – flavones, flavonol, flavanone, isoflavone, flavan-3-ol, and anthocyanidins were screened for inhibitory activity on α-amylase and α-glucosidase enzymes and the chemical structures responsible (structural activity relationship) for these activities were evaluated. The basic structure of flavanoid consists of Benzopyran (A & C rings) and Phenyl group (B ring). The six groups of flavonoid are classified on the basis of variation in C ring and linkage between the benzopyran and phenyl groups. Inhibitory activities of 4-hydroxylated, 4,5-dihydroxylated and 3,4,5-trihydroxylated flavonoids in the same flavonoid group were compared and it was found that activity was increased with increase in number of hydroxyl group on the B ring. The inhibitory activity was found to be increased by the unsaturated C ring, 3-OH, 4-CO, linkage of B ring at position 3, hydroxyl substitution on B ring. E.g. 2,3-double bond (isoflavone, flavones, and flavonol>flavanone and flavan-3-ol), 5-OH of flavonol or isoflavone (quercetin>fisetin; genistein>daidzein), linkage of B ring at 3 position (genistein>apigenin) and hydroxyl substitution on B ring increased the inhibitory activity (genistein>luteolin). It was found that A, B and C rings structures were related to inhibitory activity (69).

Glycosides: Chrysophanol-8-O-β-D-glucopyranoside and chrysophanol anthraquinones from *Rhubarb* rhizome showed good antidiabetic properties (70). Rhaponticin and rhein isolated from *Rhei Rhizoma* improved glucose tolerance by inhibiting α-glucoamylase activity, increasing insulin sensitivity and delaying carbohydrate digestion in *STZ-induced* diabetic mice. *In vitro* studies also showed improvement in insulin sensitivity (71). Dolichandroside A, a new phenylpropanoid glycoside isolated from *Dolichandrone falcate* Seem, is a novel α-glucosidase inhibitor, while saponarin II, isolated from the same plant, is very effective α-glucosidase inhibitor having the same potency as acarbose (72). *Dendrobium chrysotoxum* Lindl. is a traditional Chinese herb. Polysaccharides isolated from this plant have been found to significantly reduce blood glucose levels in alloxan-induced diabetic mice as well as having good antioxidant activity (73). Lupinoside isolated from *Pureria tuberosa* helps in prevention of palmitate-induced impairment of insulin (74).

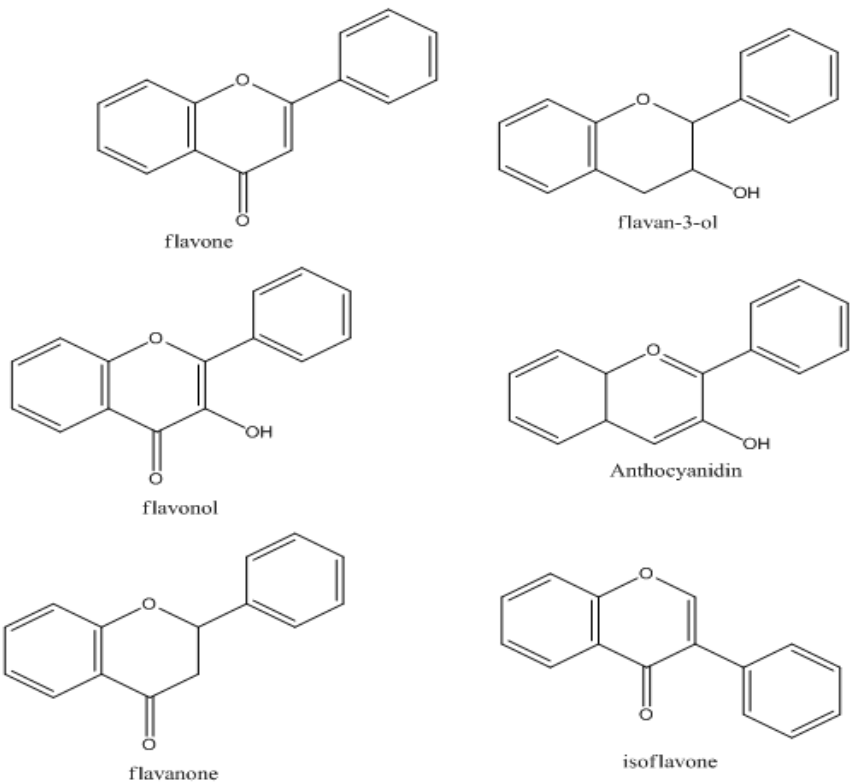

Figure 2. Structures of six groups of flavonoids

Alkaloids: *Adhatoda vasica* Nees, a common Indian Ayurvedic plant, was screened for activity against α-glucosidase and α-amylase. The aqueous methanolic extract of its leaves showed high sucrase inhibitory activity and enzyme assay-guided fractionation led to discovery of vasicine and vasicinol. Both the compounds showed high sucrase inhibitory activity by reversible inhibition of sucrose hydrolyzing activity of rat intestinal α-glucosidase. The enzymatic inhibition of α-glucosidase was studied for the first time in this plant, although it is well known for other pharmacological activities (75). Two new active

compounds, uniflorines A and B, have been isolated from the leaves of *Eugenia uniflora* L. and showed reduction in plasma glucose levels in sucrose tolerance tests on mice and inhibited α-glucosidase enzyme (76).

Essential oils: A mixture of oleanolic acid and ursolic acid in a ratio of 2:1 isolated from *Phyllanthus amarus* was screened for α-amylase inhibition and found to exhibit significant inhibitory activity (77). Roselle tea extract is made from the dried flowers of *Hibiscus sabdariffa* Linn. and is a popular beverage in Thailand. Hibiscus acid and its 6-methyl esters isolated from a Roselle tea extract showed significant inhibition of porcine pancreatic amylase (78). The essential oils from the wood of *Juniper oxycedrus* showed good activity against α-amylase, while the wood and berries of the same plant possess significant antioxidant activity (79). A diterpenoid, andrographolide isolated from an ethanolic extract of *Andrographis paniculata* (Burm.f.) Nees (Acanthaceae) showed significant α-glucosidase inhibition (80). Swietenine, a tetranortriterpenoid, isolated from *Swietenia macrophylla* seeds showed significant *in vivo* hypoglycemic and hypolipidemic activity in Type II diabetic rats (81).

Two new compounds, 7'-(3', 4'-dihydroxyphenyl)-N-((4-methoxyphenyl) ethyl) propenamide and 7'-(4'-hydroxy, 3'-methoxyphenyl)-N-((4-butylphenyl) ethyl) propenamide, isolated from *Cuscuta reflexa* ROXB showed strong inhibition for α-glucosidase (82). Pipataline, pellitorine, sesamine, brachystamide B and guineensine were isolated from *Piper longum* by bio-activity (α-glucosidase enzyme inhibition) guided fractionation and found to possess potent inhibitory activity (83).

From the scientific evaluation of phyochemicals, it is clearly seen that the majority of foods traditionally used to reduce hyperglycemia and related disorders (obesity etc) are rich in polyphenolic compounds and flavonoids.

Conclusion

As α-amylase is a key enzyme for starch hydrolysis and α-glucosidase for intestinal absorption, these enzymes help in digestion and uptake of carbohydrates. Inhibition of these enzymes significantly decreases the postprandial increase of blood glucose level after a mixed carbohydrate diet and can therefore be an important strategy in the management of hyperglycemia linked to Type II diabetes. Currently available drugs, acarbose and voglibose, which inhibit these enzymes, have associated side effects of abdominal distention, flatulence, meteorism and diarrhea, which might be caused by the excessive inhibition of pancreatic α-amylase resulting in the abnormal bacterial fermentation of undigested carbohydrates in the colon. Natural inhibitors from dietary sources have shown lower inhibitory effects against α-amylase activity and stronger inhibitory activity against α-glucosidase, which can be a good strategy to reduce postprandial hyperglycemia with minimal side effects (24).

Lifestyle modifications and proper diet management are also important factors in the treatment and prevention of diabetes mellitus and its related complications. Diabetes patients should include wholegrain products, vegetables, fruits, low fat milk, food high in fibre, meat products and other appropriate sources of proteins, soft margarines and vegetable oils rich in

monounsaturated fatty acids and foods with low glycemic index in their diets (84). Nuts and peanuts are beneficial in maintaining glucose and insulin homeostasis. Omega-3-fatty acids and regular consumption of fish helps in diabetes by reducing the chances of getting cardiovascular diseases and cinnamon may also have some affect in reducing blood glucose (85). Exercise and physical activity are the other important factors to manage diabetes to increase energy expenditure, as physical inactivity and a sedentary lifestyle are associated with metabolic disorders such as diabetes, obesity and other cardio-vascular disorders (86). Yoga, which is an ancient Indian exercise, is very good for stress management, and increases mental discipline, voluntary control of autonomic nerves and relaxation. It is practiced by muscles stretching, breathing exercises, behavioral modification, and diet control through mental discipline, all of which are suggested to help in diabetes management (16). Tai-chi, a Chinese martial arts which helps to maintain health and longevity, has also proven to be beneficial in diabetes management (87).

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Chapter 6

Biomolecules with Anti-Mycobacterial Activity

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Abstract

Tuberculosis is an infectious, primary pulmonary disease, caused by *Mycobacterium tuberculosis* that remains an important public health problem worldwide with approximately nine million new cases and two million deaths per year. TB is considered the most important disease caused by a single infectious agent and its control has been difficult due to the lack of an effective vaccine, association with HIV infection and the progressive development of resistance to anti-TB drugs.

Alternative anti-mycobacterial drugs are urgently needed; studies have shown that medicinal plants traditionally used to treat respiratory diseases are a potential source of new and efficient compounds to treat tuberculosis.

In this chapter we will describe some compounds, found in plants that have been tested in different bioassays and showed anti-mycobacterial activity.

Introduction

Tuberculosis (TB) is a major cause of illness and death worldwide, especially in Asia and Africa. Globally, 9.2 million new cases and 1.7 million deaths from TB occurred in 2006, of

which 0.7 million cases and 0.2 million deaths were in HIV-positive people. Overall, an estimated one third of the world population is infected with *Mycobacterium tuberculosis*. Population growth has boosted these numbers compared with those reported by the World Health Organization (WHO) for previous years. More positively, and reinforcing a finding first reported in 2007, the incidence rate appears to have been falling globally since 2003. The African, South-East Asia and Western Pacific regions accounted for 83% of total case notifications. [WHO Report 2008; LoBue et al, 2009; Garzón et al, 2008].

Tuberculosis is produced by *Mycobacterium tuberculosis*, microorganism belonging to the *M. tuberculosis* complex which also includes *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. microti*, and *M. canneti*; more recently the smooth African varieties named *M. prototuberculosis* had been added to this complex.

Tuberculosis is acquired through the respiratory way, and the most common form is the primary pulmonary infection, although it can affect almost any other organ including kidneys, brain, skin, etc. Pulmonary disease is clinically characterized by cough, fever, chills, and in advanced cases, hemoptisis.

This disease is preventable and curable, but infected people can die if they do not get proper treatment. Therapy was started with the introduction of streptomycin as monotherapy in 1943, however after a few years of use there was the appearance of many resistant isolates. Isoniazide (INH) was introduced in 1952 and in the 1970´s, rifampn reached the market [Espinal & Salfinger; 2005]. The use of rifampin (RIF) allowed the shortening of the therapy from 18 to 9 months. In the 1980´s the period of therapy was decreased even more with the use of pyrazinamide (PZA) to 9 to 6 months which constitutes the currently therapeutic scheme used. In theory, the infection can be cured completely with RIF-INH-PZA, however, the complete scheme has to be taken for several months and in many cases, that produces low adherence to therapy resulting in the development of resistant isolates; the association with HIV infection has complicated even more this situation [WHO, 2008b]. The influence of HIV infection on susceptibility to develop active TB in infants is even worse, Hesseling et al [2009] reported in a study performed in South Africa that incidence of tuberculosis in HIV-infected infants was 1596 cases per 100,000 population against 65.9 per 100,000 among HIV-uninfected infants. This also may represent a source of MDR strains as many of these people do not have the adequate access to anti-TB drugs.

The appearing of resistant isolates to the best two anti-tuberculous drugs, rifampin and isoniazide (MDR-TB), has prompted the use of more toxic and less effective second line compounds such as capreomycin, cycloserin, ethionamide, kanamycin, ofloxacin, PASER, and prothionamide [WHO, 2008]. Extensively drug-resistant tuberculosis (XDR-TB) is a type of multidrug-resistant TB. XDR-TB is defined as a tuberculosis case resistant to isoniazid and rifampin, plus to any fluoroquinolone, and at least to one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin). Since XDR-TB isolates are resistant to first- and second-line drugs, the patients are left with treatment options which are much less effective. XDR-TB cases are of special concern for persons with HIV infection or other conditions with a debilitated immune system [CDC, 2008].

The spread of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant TB (XDR-TB) is a major medical and public health concern for the world. These two forms

of highly drug-resistant TB threaten to make TB into an untreatable and highly fatal disease, particularly in resource-poor countries with a high prevalence of AIDS [Chan et al, 2008].

No new classes of specific drugs for TB have been developed in the past 30 years and the global number of TB cases is still increasing. Therefore there is an urgent need to develop faster acting and effective new anti-tubercular agents in order to control this infection. In this chapter we will describe some compounds found in plants that have been analyzed in different bioassays and showed anti-mycobacterial activity.

Anti-TB Compounds Derived from Plants

Traditional medicine, particularly the use of plants or their extracts, has been the most important source for the screening and isolation of natural products with anti-mycobacterial activity. Use of traditional medicine for treatment of respiratory diseases, including tuberculosis, has been a source for many potential opportunities to find new anti-TB drugs. Several groups around the world have analyzed plants used by people as herbal medicines, looking for biological activities against infections and other illness. To date, only a few compounds with significant anti-mycobacterial activity have been isolated, and most of these compounds are in the phase of *in vitro* laboratory or animal models testing.

Kolodziej et al [2003] reported the antibacterial activity of *Pelargonium sidoides*, plant species used in folk medicine by the Southern African native population. *P sidoides* extracts contained oxygenated coumarins and simple phenol gallic acids [Kayser et al, 1997]. Another medicinal plant from southern Africa used to treat chronic infections is *Carpobrotus* sp that by bio-autography, together with thin layer chromatography (TLC) analyses showed anti-bacterial activity [Springfield et al, 2003]. Bamuamba et al [2008] analyzed five African plant species for anti-mycobacterial activity. They found that extracts of *Buddleja saligna* and *Leysera gnaphalodes* exhibited significant anti-mycobacterial activity, primarily associated with the presence of non-cytotoxic triterpenoids oleanolic acid in *B. saligna* and both oleanolic and ursolic acids in *L. gnaphaloides*. In fact, there are many plants used against TB in African traditional medicine. McGaw et al [2008] summarize the available knowledge on South African plants used to treat TB symptoms, and anti-mycobacterial efficacy of plant-derived extracts and compounds.

The activity of cryptolepine hydrochloride, a salt of the main indoloquinoline alkaloid from the West African medicinal plant *Cryptolepis sanguinolenta*, was assessed against the fast growing mycobacterial species *M. fortuitum, M. phlei, M. aurum, M. smegmatis, M. bovis* BCG and *M. abcessus* and the MICs ranged over 2-32 μg/mL [Gibbons et al, 2003].

Ethiopian medicinal plants used to treat various infectious diseases were assessed for their possible activity against TB *in vitro*. The authors studied fifteen crude extracts prepared from seven plants. Only the acetone fraction obtained from the stem bark of *Combretum molle* showed inhibitory activity at 100 μg/ml. Phytochemical analysis of the bioactive fraction led to the isolation of two major tannins identified as ellagitannin and punicalagin. The last compund was found to inhibit totally the growth of *M. tuberculosis* (ATCC 27294) and a clinical isolate fully sensitive to the standard antituberculosis drugs [Asres et al, 2001].

Tibetan traditional medicine reports the use of *Gentianopsis paludosa* against mycobacteria. At least three dimethoxyxanthones extracted from *G. paludosa* have shown *in vitro* growth inhibitory effects on mycobacteria [Yeung et al, 2009]. Compounds as 1-O-beta-d-glucopyranosyl-5-hydroxy-3-methoxyxanthone,1-O-[beta-d-xylopyranosyl- (1 --> 6)-beta-d-glucopyranosyl]-7,8-dihydroxy-3-methoxyxanthone, and apigenin have been identified from this plant [Wang et al, 2007].

In a screening of plants from New Caledonia and Vanuatu, looking for antimicrobial activity, Billo et al [2005] examined 55 extracts of 21 plants, including an endemic species: *Amborella thricopada*. They found inhibitory activity against *Mycobacterium bovis* BCG strain at a concentration of 100 mg/ml of the methanolic and dichloromethane extracts of *Amborella trichopoda*, *Codiaeum peltatum*, *Myristica fatua*, and essential oils of *Myoporum crassifolium*. While a very promising activity was found in the methanolic extract of *Amborella trichopoda* fruits with a MIC of 1- 2.5 µg/ml.

Gautam et al [2007] reviewed 255 Indian plant species from a wide range of families that have shown *in vitro* anti-mycobacterial activity and a number of active plant-derived compounds belonging to different chemical classes that have been isolated. In this review, the authors enumerated in a practical format the plants, plant part used, type of extract and in vitro activity (MIC value) reported since 1935.

A very extensive work in a region, in order to find compounds with anti-mycobacterial activity was performed by Graham et al [2003] on 270 Peruvian plants of 63 families. In this study, half of the samples showed greater than 50% growth inhibition of *M. tuberculosis* at 50 µg/ml. The most promising were *Senna silvestris* (MIC: <6.25 µg/ml) and *Sommera sabiceoides* (MIC: <6.25 µg/ml).

There are many plants in Mexican traditional medicine that have been used by empirical knowledge against pulmonary diseases, including reports of plants to treat specifically TB [Aguilar et al, 1998]. Studies on Mexican plants have produced several interesting results with promising compounds to be considered for further analysis on their activity anti-TB. Three pentacyclic triterpenoids with oleanane nucleus, together with beta-sitosterol have been isolated from *Lantana hispida*. The molecular structures of the compounds were characterized as 3-acetoxy-22-(2'-methyl-2Z-butenyloxy)-12-oleanen-28-oic acid (1), 3-hydroxy-22beta-(2'-methyl-2Z-butenoyloxy)-12-oleanen-28-oic acid (reduced lantadene A) (2) and oleanolic acid (3). Minimal inhibitory concentration (MIC) values for compounds 1 and 2 were 50 µg/ml, and for compound 3 the MIC was 25µg/ml. [Jimenez-Arellanes et al, 2007]. Glycolipids, sesquiterpenoids and triterpenoids have been isolated from selected mexican medicinal plants. Although the tested compounds showed moderate antimycobacterial activity (the MIC values ranged from 16 to 128 µg/mL), their presence in the analyzed plant species supported the rationale for their traditional use in the treatment of tuberculosis [Rivero-Cruz et al, 2005]. Other studies are taking the first steps by evaluating different plant extracts; Camacho-Corona et al [2008] screened nine Mexican plants and they found that *Nasturtium officinale, Citrus sinensis, Citrus aurantifolia, Foeniculum vulgare, Larrea tridentata, Musa acuminata and Olea europaea* extracts produced inhibitory activity against drug sensitive *M. tuberculosis* and also against drugs-resistant isolates of *M. tuberculosis*. In the other hand, Cruz-Vega et al [2008] showed that extracts from plant samples collected in Mexico of *Juglans regia*, *Juglans mollis*, *Carya illinoensis*, and

Bocconia frutescens showed anti-*M. tuberculosis* activity. Also, the evaluation of 25 ethanol extract of plants used in the traditional medicine of Baja California Sur (Mexico) were tested for anti-*M. tuberculosis* activity from these, ten extracts showed activity at 100 μg/ml [Murillo-Alvarez, 2001]. Hexane and acetone extracts of *Flourensia cernua* DC showed a MIC of 50 and 25 μg/mL against sensitive and resistant strains, respectively. The extracts not only inhibited the growth but killed *M. tuberculosis* [Molina-Salinas et al, 2006]. Although, no compound has been isolated from all those plants yet.

Chemical exploration of *Camchaya calcarea* (Compositae) has led to the isolation of nine known sesquiterpene lactones. Seven of them exhibited potent antimycobacterial activity [Vongvanich et al, 2006].

Lack of maturation of phagosomes containing pathogenic *M. tuberculosis* within macrophages has been widely recognized as a crucial factor for the persistence of mycobacterial pathogen. Host molecule tryptophan-aspartate containing coat protein (TACO) has been shown to play a crucial role in the arrest of such a maturation process. The down-regulation of TACO gene expression by epigallocatechin-3-gallate polyphenol from green tea was accompanied by inhibition of mycobacterium survival within macrophages as assessed through flow cytometry and colony counts [Anand et al, 2006].

Three sterol compounds from *Thalia multiflora* named stigmast-5-en-3beta-ol-7-one, stigmast-4-ene-6beta-ol-3-one, stigmast-5,22-dien-3beta-ol-7-one, and stigmast-4,22-dien-6beta-ol-3-one, exhibited antimycobaterial activity with MIC values of 1.98, 4.2 , 1.0, and μg/mL, respectively [Gutierrez-Lugo et al, 2005].

The dichloromethane extract of stem bark of *Warburgia ugandensis* afforded three new coloratane sesquiterpenes, and nine known sesquiterpenes. Some of them were active against *M. aurum, M. fortuitum, M. phlei* and *M. smegmatis*; with MIC values ranged from 4 to 128 μg/ml compared to ethambutol (MIC range, 0.5 to 8 μg/ml) and isoniazid (MIC range, 1 to 4 μg/mL) [Wube et al, 2005].

Searching for new drugs that are effective against MDR strains of *M. tuberculosis* and can augment the potential of existing drugs against tuberculosis, Bapela et al [2006] made combinations of naphthoquinone, 7-methyljuglone, isolated from the roots of *Euclea natalensis*, with isoniazid or rifampicin and resulted in a four to six-fold reduction in the minimum inhibitory concentration of each compound. Fractional inhibitory concentration (FIC) indexes obtained were 0.2 and 0.5, respectively, for rifampicin and isoniazid, suggesting a synergistic interaction between 7-methyljuglone and these anti-TB drugs.

Other compounds under research for their anti-Tb activity include the following: From the root of *Calliandra californica* two new cassane-type diterpenes were isolated and characterized, escobarine A and B, which showed promising activities against two *M. tuberculosis* strains [Encarnacion-Dimayuga et al, 2006]. Compounds isolates from the root of *Garcinia linii*, 1,7-dihydroxy-3-methoxyxanthone and 1,5-dihydroxy-3-methoxyxanthone showed antitubercular activities with MICs of 3.1, and 6.3 μg/mL against *M. tuberculosis* [Chen et al, 2006]. Callicarpic acid B, 12-Deoxy-11,12-dihydro-seco-hinokiol methyl ester, and alpha-tocopherol trimer B isolated from the leaves and twigs of *Callicarpa pilosissima* exhibit antitubercular activities (MICs $\leq$ 63.6 μM) against *M. tuberculosis* H37Rv *in vitro* [Chen et al, 2009]. Erythrophloin C and suberosol B roots of *Beilschmiedia erythrophloia,* had MIC values of 50 and 28.9 mg/mL against *M. tuberculosis* H37Rv [Yang et al, 2009].

Celahin C and salasol A isolated from the root of *Microtropis japonica* exhibited *in vitro* anti-tuberculosis activity, both with an MIC value of 15.0 mg/ml against *M. tuberculosis* H37Rv [Chou et al, 2008]. Ursolic acid, squalene and farnesol isolated from *Chamaedora tepejilote* hexane extract produced a *M. tuberculosis* growth inhibition of 99% at a concentration of 100 µg/mL [Jimenez et al, 2005].

Alkaloids and lactones also show anti-mycobacterial activity: Anti-TB bioassay-directed fractionation led to the isolation of carbazole alkaloids, as well as the gamma-lactone derivative of oleic acid, from the CH_2Cl_2 extract of the stem bark of *Micromelum hirsutum*. The lactone derivative of oleic acid, (-)- Z-9-octadecene-4-olide showed potent in vitro anti-TB activity against H37Rv (MIC: 1.5 µg/mL), and exhibited activity against the Erdman strain of *M. tuberculosis* in a J774 mouse macrophage model (EC_{90}: 5.6 µg/mL). The carbazoles include the new micromeline and known alkaloids: lansine, 3-formylcarbazole, and 3-formyl-6-methoxycarbazole, had anti-TB MIC values between 14.3 and 42.3 µg/mL [Ma et al, 2005].

Constituents from the roots of *Engelhardia roxburghiana*, Engelhardione, 3-methoxyjuglone, and (-)-4-hydroxy-1-tetralone showed antitubercular activities with MIC values of 3.125, 3.125, and 6.25 microg/mL against three different strains of *M. tuberculosis*, and with MIC values of 0.2, 0.2, and 4.0 µg/mL against *M. tuberculosis* H37Rv [Lin et al, 2005].

Antitubercular bioassay-guided fractionation of the n-hexane and CH_2Cl_2-soluble extracts of above-ground biomass and roots of *Valeriana laxiflora* led to the isolation of a new lignan (+)-1-hydroxy-2,6-bis- epi-pinoresinol, along with eleven known including compounds, betulin, betulinic acid, 5,7-dihydroxy-3,6,4'-trimethoxyflavone, 23-hydroxyursolic acid, oleanolic acid, tricin, and ursolic acid. In a microplate alamar blue assay against *Mycobacterium tuberculosis*, compounds exhibited MICs of 15.5 - 127 µg/mL [Gu et al, 2004].

Kanokmedhakul et al [2003], isolated from the mushroom *Scleroderma citrinum* the compound 4,4'-dimethoxyvulpinic acid and two of its derivatives, the dibromo derivative 5 and acetate derivative 6; all of them exhibited inhibitory activity towards *M. tuberculosis*. Limmatvapirat et al, [2004] isolated from the aerial parts of *Abrus precatorius*, a known isoflavanquinone, the abruquinone B which exhibited antitubercular activity.

From the hexane-soluble fraction of an ethanol extract from leaves and stems of *Stemodia foliosa* (Scrophulariaceae), the new stearic acid 4-[(n-pentoxy)phenethyl] ester was isolated and exhibited antibacterial properties at 10 µg/mL concentration by using disc diffusion method against the fast-acid bacterium *M. fortuitum*. [Dantas da Silva et al, 2002].

Cantrell et al [2001] presented a review about reports, up to that year, on plant-derived terpenoids showing moderate to high activity in *in vitro* bioassays against *M. tuberculosis*. In that review, mono-, sesqui-, di- and triterpenes, and sterols, their structural analogs and semisynthetic derivatives are discussed, with particular emphasis on the structural features essential for anti-mycobacterial activity.

There are some well known and characterized compounds from plants extracts that present relevant activity anti-TB; Luteolin isolated from methanol extracts of *Ficus chlamydocarpa* (FCR) and Ficus cordata showed activity against *M. tuberculosis* exhibiting a MIC of 4.88 µg/ml (Kuete, 2008). Norditerpenoid 12-demethylmulticauline from *Salvia*

multicaulis with a remarkable MIC of 0.46 µg/mL and its C-12 methoxy analog, with a MIC of 5.6 µg/mL [Ulubelen et al, 1997]. Several diterpenoides and triperpertenoides had shown anti-mycobacterial activity; ergosterol-5,8-endoperoxide from *Ajuga remota* with a MIC of 1 µg/mL [Cantrell et al,1999b]. Pentacyclic triterpenoids isolated from *Sarmienta scandens* like zeorin, and isolated by bioassay-guided fractionation showed a MIC of 8 µg/mL [Wachter et al, 1999].

The diterpene (E)-phytol isolated from *Lucas volkensii* showed potent *in vitro* activity against *M. tuberculosis* (MIC 2 µg/mL). In addition, the analogs (Z)-phytol and (3R,S,7R,11R)-phytanol demonstrated MICs of 2 µg/mL, suggesting that the 2,3-double bond may not be essential for bioactivity. However, (E)-phytyl acetate and (E)-phytol methyl ether showed MICs of 16 µg/mL /ml implying that a free hydroxy group, as present in (E)-phytol, is required for significant activity [Rajab et al, 1998]. Triterpenoids isolated from the non-saponifiable lipid fraction of the flower extract of chrysanthemum (*Chrysanthemum morifolium*) were tested for their antitubercular activity against *M. tuberculosis* strain H37Rv using the Microplate Alamar Blue Assay (MABA). Fifteen compounds exhibited the highest activity with a MIC in the range of 4-64 µg/mL, among which were maniladiol (MIC 4 µg/mL), 3-epilupeol (4 µg/mL), and 4,5-alpha-epoxyhelianol (6 µg/mL) [Akihisa et al, 2005].

Bioactivity-guided fractionation of the CH_2Cl_2/MeOH extract of the aerial part of *Ruprechtia triflora Griseb*. led to the identification of several sterols and a triterpene as the active components against *M. tuberculosis*. In a microplate alamar blue assay, sterols from *R. triflora* were found to be active with MIC values ranging from 2 - 128 µg/mL, with 5alpha,8alpha-epidioxyergost-6,22-dien-3beta-ol, 5alpha,8alpha-epidioxystigmasta-6,22-dien-3beta-ol and stigmast-4-en-6beta-ol-3-one being the most active, each with an MIC value of 2 µg/mL. Among the diterpenes from *C. pinnifolia*, 19-malonyloxydehydroabietinol and 19-methylmalonyloxy- ent-isopimara-8(9),15-diene were most active each with an MIC value of 4 µg/mL. MIC values for the triterpenes 3-epi-ursolic acid and 3-epi-oleanolic acid from *C. pinnifolia* were determined to be 8 and 16 µg/mL, respectively [Woldemichael et al, 2003]. A similar approach for CH_2Cl_2/MeOH extraction of the aerial parts of *Sapium haematospermum*, produced a new pimarane, and a highly oxygenated novel chalconoid, (3 alpha-hydroxyolean-12-ene) and (cycloartanol) which were active against *M. tuberculosis* with MIC values of 4, 12.2, 13.4, and 8 µg/mL respectively [Woldemichael et al, 2004].

Bioactivity-guided investigations of methanolic extracts of seeds of *Melia volkensii* resulted in the isolation of two new euphane (20R)-type triterpenoids: 12b-hydroxykulactone and 6beta-hydroxykulactone, both of them are derivatives of kulactone. Another isolated compound was kulonate. Compounds 12b-hydroxykulactone and kulonate had MICs of 16 µg/mL, while 6b-hydroxykulactone was more active with an MIC of 4 µg/mL [Cantrell et al, 1999a].

Constituents isolated from aerial parts of *Junellia tridens*, 3-Epioleanolic acid and oleanonic acid showed antitubercular activities with MIC values of 16 µg/mL against *M. tuberculosis* [Caldwell et al, 2000].

Compounds isolated from methanolic extracts of *Commiphora mukul*, *Psoralea corylifolia* and *Sanguinaria canadensis* were found to have antimycobacterial activity against *M. aurum* only (MIC=62.5 µg/mL). Bioassay guided fractionation led to the isolation of two known benzophenanthridine alkaloids, sanguinarine and chelerythrine from the roots of *S.*

canadensis and the known phenolic meroterpene, bakuchiol from the seeds of *P. corylifolia*. Chelerythrine was the most active against *M. aurum* and *M. smegmatis* (IC50=7.30 µg/mL [19.02 µg/mL] and 29.0 µg/mL [75.56 µg/mL], respectively) [Newton et al, 2002].

A crude ethanol extract and hexane fraction from *Morinda citrifo*lia Linn. (Rubiaceae) showed antitubercular activity. The major constituents of the hexane fraction were E-phytol (MIC: 32 µg/mL), cycloartenol (MIC: <64 µg/mL), stigmasterol (MIC: 32 µg/mL), B-sitosterol (MIC: 128 µg/mL), campesta-5,7,22-trien-3B-ol (MIC: 2.5 µg/mL) and the ketosteroids stigmasta-4-en-3-one and stigmasta-4-22-dien-3-one. E-Phytol, a mixture of the two ketosteroids, and the epidioxysterol derived from campesta-5,7,22-trien-3B-ol all showed pronounced antitubercular activity (MIC: < 2.0 µg/mL) [Saludes et al, 2002].

Following bioassay-guided fractionation, phytosterol saringosterol was isolated from *Lessonia nigrescens* as the active component for anti-mycobacterial activity. The MIC values for saringosterol and its 24S and 24R epimers were determined as 0.25, 1, and 0.125 µg/mL [Wachter et al, 2001]. With the same type of assay, ostruthin (6-geranyl-7-hydroxycoumarin) was isolated from the roots of *Peucedanum ostruthium Koch* (Apiaceae) as a compound with high in vitro activity against several species of rapidly growing mycobacteria, namely *M. abscesus*, *M. aurum*, *M. fortuitum, M. phlei* and *M. smegmatis*. Minimum inhibitory concentrations ranged between 3.4 to 107.4 µM and were comparable to those of ethambutol and isoniazid [Schinkovitz et al, 2003].

Nine flavonoids (1–9) have been isolated from *Kaempferia parviflora*. Among these, 5,7,49-trimethoxyflavone and 5,7,39,49-tetramethoxyflavone showed mild antimycobacterial activity with the minimum inhibitory concentrations (MIC) of 200 and 50 µg/mL, respectively [Yenjaia et al, 2004].

Conclusion

As seen, a great perspective to obtain new anti-mycobacterial drugs emerge from basic compounds isolated from plants. Most of the compounds have been found following ethnobotanical criteria, this is the traditional and ancient knowdelge of the use of the plants to fight different illness. In the case of tuberculosis, many plants studied are those that are used to treat pulmonary disease or its symptoms including cough, fever, and expectoration.

Some molecules have been already analyzed for activity against mycobacteria, either as growth inhibitor or bactericidal. But a wide and enormous diversity of plants are still waiting to be investigated to find new molecules. A key on the discovery and development of new drugs anti-TB is to study the bacterial enzymes and metabolic pathways to find analogous molecules in nature, either from plants or other organisms that block or inhibit the enzyme function or an especific metabolic pathway. Researchers also have the option to modify by chemical methods the molecules to make them more active. Molecular modifications may include demethylation, hydroxylation, sulfation, and ribosylation (Yuan, 2006). Once a good drug candidate has been discovered, the modification of the original compound must be designed to have an active molecule easily administrated, with the adequate solubility to reach the places where the bacteria is usually found and then directly interfere on very

essential cellular mechanisms for a pathogen like *M. tuberculosis*: virulence genes function, membrane permeability and transport of *M. tuberculosis* as target of new drugs.

Certainly, we need new drugs, more effective, less toxic, with a shorter therapy scheme and also more affordable for undeveloped countries. But, to find a new drug is still just a step to reach the control of tuberculosis worldwide. It is also needed an international collaboration to build a better health and educational system. It seems we are still behind.

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Chapter 7

Metal Concentrations in Taiwanese Health Food, *Angelica Keiskei*, Other Products and Maximum Daily Intake

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1. Abstract

"*Angelica keiskei* AK", a health food, originated from Japan (*Umbelliferae*, "Ashita-Ba" in Japanese), has been distributed islandwide and widely consumed by the general public in Taiwan during the past twenty-five years. This plant was recognized as natural aromatic and an important medicinal plant of traditional Chinese herbs. Presently, this herb is treated as a diuretic, analeptic, lactagogue and has been recommended, cultivated, and propagated by the Taiwan Agricultural Research Institute (TARI). AK was sampled from five main planted areas to ensure diversity in the summer and spring harvest seasons in central Taiwan. Epithermal and instrumental neutron activation analysis (ENAA and INAA) revealed the presence of nearly twenty metals in the roots, fresh leaves and stems of the plant, as well as in end-products such as tea bags and capsules of the Taiwanese health food product. This research employed ENAA to identify aluminum (Al), arsenic (As), bromide (Br), chloride (Cl), iodine (I), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), antimony (Sb), and samarium (Sm) and INAA to identify chromium (Cr), iron (Fe), lanthanum (La), rubidium (Rb), scandium (Sc), selenium (Se), vanadium (V) and zinc (Zn). Some of these elements are classified as either toxic or essential to humans. In the collected samples the elements exist in widely differing concentrations, ranging from 10^5 to 10^{-2} μg/g from different farms. Zinc concentrations in the tea bags are higher than those in the drinking teas, Mg, and I were the first elements to be detected. The elemental concentrations and maximum daily intake (MDI) of this herb are compared with those of *Angelica sinensis* (Danggui in Mandarin),

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Ligusticum chuanxiony (Chuanxiong in Mandarin) and *Panax ginseng* (Ginseng in Mandarin) as well as with the recommended daily dietary intake values for Taiwanese consumers, developed by the WHO. The prescription (12 g/day for adult, 6g/day for children), the MDI of As is below that recommended by WHO/FAO, and thus the average daily intake of Al, Fe and Sc in Taiwan is probably excessive. However, the MDI of Cr, Fe, Mn, and Zn among five farms and available in the markets are all below the levels recommended by WHO/RDA. Finally, the MDI of Al, Br, Cl, K, La, Na, Rb, Sb, Sc, Sm and V correlate closely with the levels recommended by WHO/RDA.

2. Introduction

"*Angelica keiskei* AK", a health food, originated from Japan (*Umbelliferae*, "Ashita-Ba" in Japanese), had been widely planted in cool and humid climates at altitudes of 400 to 1200 meters above sea level at five planted farms, Chuchi, Lalashan, Minder, Puli and Taian, all in central Taiwan shown at Figure 1.

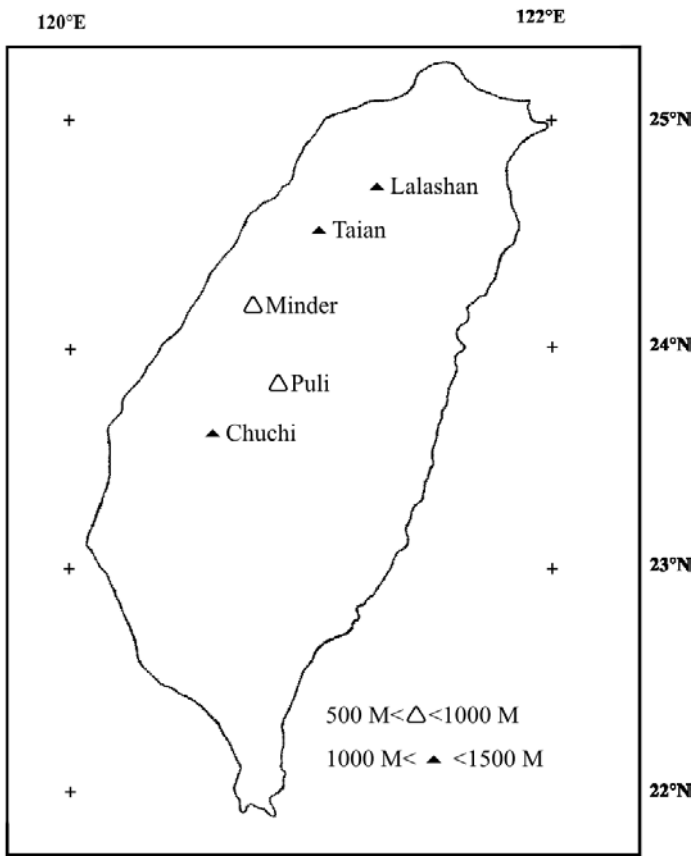

Figure 1. Five main planted areas in central Taiwan.

Each planting covers nearly 50 acres, and the plant is harvested twice each year, in the winter and spring during the past twenty-five years (Chen, 2004; Chen, 2003; Chen, 2002; Chen et al., 2002; Liu, et al., 1992). Data on this tropic are currently lacking in Taiwan.

This plant was recognized as natural aromatic and an important medicinal plant of traditional Chinese herbs (Okuyama et al., 1991). Presently, this herb is treated as a diuretic, analeptic, lactagogue and has been recommended, cultivated, and propagated by the Taiwan Agricultural Research Institute (TARI) (Chen, 1995; Fujita et al., 1992; Liu et al., 1992; Inamori et. al., 1991; Okuyama et al., 1991). Numbers of consumers are continuously expanding (Chen, 2004; Chen, 2003; Chen, 2002; Chen et al., 2002; Liu et al., 1991). The elements of AK are considered to be beneficial to human health and have been extensively investigated in recent years. It is very interesting to know the elemental concentrations presented in this health food. Reliable multi-element analytical methods must be applied to identify elements herein. ENAA and INAA are effective techniques of obtaining most of the trace elements of interest and reliable multi-element analytical methods without any pretreatment before irradiation (Chen, 2009; Chen, 2004; Chen, 2003; Chen, 2002; Chen et al., 2002; Chen and ChangLai, 2001; Chen and Pan, 2001; Wei et al., 1997; Wang et al., 1996; Ibrahim, 1995; Ibrahim, 1994; Wang et al., 1993). It is interesting to compare AK with three Chinese herbs (AS, LC and PG) all grown in humid climates and the AK root generally resemble those in the root/rhizomes of AS and LC which have been used as Chinese medicine for more than four millennia. Finally, since it helps to reveal the roles of these elements, the maximum daily intake (MDI) is also investigated for reasons of safety.

3. Experimental

3.1. Preparation of Activated Samples

For the ENAA and INAA of AK, the dried samples were packed and sealed in polyethylene (PE) bags. Raw AK, approximately 5 kg of fresh leaves and stems, and 1 kg of other products such as tea bags and capsules, were taken simultaneously from the five planted areas during the winter and spring harvests to ensure diversity, as endorsed by TARI in Taiwan. The samples had to first be washed with distilled water, then cut into chips using a stainless steel knife, and finally homogenized and freeze-dried at -40°C, under 13 mm-Hg for 2 days.

Freeze-dried AK tissues were powdered in an all Teflon cylindrical mill and sieved through a 0.9×0.8 mm sieve (Wei et al., 1997). Tea bags and capsules were dried materials, and used in their original forms after they were purchased from the five planted areas. In addition, each sample was then double sealed into another PE bag and preserved in a desiccator before further irradiation. AK was weighted 350±10 mg for ENAA and 150±10 mg for INAA. A weighed sample was packed into 3×3 cm^2 PE bag then doubly sealed into another PE bag for irradiating in the vertical tube (VT) irradiation positions for long irradiation and pneumatic tube (PT) irradiation positions for short irradiation in the Open pool Reactor of Tsing Hua University (THOR). Each bag was subsequently enveloped in a host PE to prevent pre-irradiation contamination. The reference material used herein, lichen

(IAEA 336), also weighing around 150 and 350 mg, was irradiated together with AK to quantify the elemental concentrations in the samples. An empty PE bag of identical size was taken as blank correction also double-sealed. Each sample and standard was prepared in triplet to minimize the statistical uncertainty as published everywhere (Chen and ChangLai, 2001; Chen and Pan, 2001; Wei et al., 1997). Blank correction for ENAA and INAA methods were also performed to determine possible interference. Each irradiated sample was paired with a 10 mg Ni-foil monitor used for neutron flux fluctuation (Chen, 2004; Chen, 2002; Chen and Pan, 2001).

3.2. Irradiation Filters and Irradiation Schemes

The larger boron-polyethylene (BPE) flexible shield cylinder container with a 164 mm H×44 mm D with 3.2 mm wall thickness (Flex/Boron, Reactor Experiments, UK) and smaller cadmium cylinder container with a 40 mm H×25 mm D with 1 mm wall thickness filter were used to screen thermal neutrons herein. Three irradiation schemes were developed: (a) short irradiations (t_i = 10 min) wrapped with Cd filter in the PT; (b) long irradiations (t_i = 24 h) wrapped with BPE filter in the VT; (c) long irradiation (t_i = 24 h) but without any wrapped in the PT. All of the irradiation schemes and decay properties for the interested nuclides are illustrated in Table 1. Details of the experimental conditions for ENAA and INAA are given elsewhere (Chen, 2009; Chen, 2004; Chen, 2003; Chen, 2002; Chen and Pan, 2001; Shirley and Lederer, 1978).

3.3. Counting and Analysis

The γ-ray spectra were measured using a calibrated HPGe detector with 15% relative efficiency. The counting system uses herein provided an energy resolution of 2.5 keV at 1333.2 keV of ^{60}Co. γ-ray spectra were analyzed by Micro SAMPO90 software coupled with personal computer connecting to a System-100 multichannel analyzer board for spectral acquisition (Chen, 2009; Chen, 2004; Chen, 2003; Chen, 2002; Chen and Pan 2001). Statistical errors in each of these values did not exceed 15% and dead times were kept below 10% (Chen and Pan, 2001; Wei et al., 1997). The difference in Mg measurement may be attributed to inaccurate determination caused by the interference of ^{56}Mn during the γ-ray measurement by ENAA. Even if the deduction of multiple γ-rays 843.8 and 846.6 keV for ^{28}Mg and ^{56}Mn, respectively, is resolvable using Micro SAMPO90 software, the relative high concentration of ^{56}Mn to ^{28}Mg in the herb samples can still decrease the statistical control of Mg in INAA measurement.

Activities of ^{80}Br and ^{128}I had less relevance to the attenuated thermal neutron in ENAA, whereas alternately, activities of ^{24}Na, ^{38}Cl and ^{28}Al were suppressed easily with the attenuated thermal neutrons. In addition, there was another kind of interfering γ-peak at 439.9 keV to the interested gamma-peak 442.9 keV from ^{128}I in this work. This unwanted gamma-peak was generated by the ^{23}Na nuclide absorbing fast neutrons with a half-life of 37.2 second to give a proton and ^{23}Ne. Since, it exists with such a comparatively short half life; it can be easily suppressed by a longer cooling time. Further, the irradiated samples also had to be cooled for at least 10 minutes for avoiding a too high Compton scattering plateau on

which the γ-peaks of interest are situated. This Compton scattering plateau is mainly generated by the 1778.8 keV γ-peak of ^{28}Al (Shirley and Lederer, 1978). In contrast, the ENAA technique alters the γ-ray spectrum noticeably within the same activated sample. That is, via the ENAA technique, either ^{128}I or ^{80}Br is identified clearly from the γ-ray spectrum (Chen et al., 2003).

Table 1. Experimental parameters and irradiation positions for ENAA and INAA in this work (the ^{65}Ni flux monitors are used for neutron fluence measurement)

Neutron shield	Irradiation position	Element	Nuclear reaction	Half-lives [a]			γ-Ray energy (keV)	
ENAA								
Cd	PT [b]	Al	^{27}Al (n, γ) ^{28}Al	2	.24	m	1778	.8
BPE	VT [c]	As	^{75}As (n, γ) ^{76}As	26	.32	h	559	.2
Cd	PT [b]	Br	^{79}Br (n, γ) ^{80}Br	16	.7	m	616	.7
Cd	PT [b]	Cl	^{37}Cl (n, γ) ^{38}Cl	37	.2	m	1642	.7
Cd	PT [b]	I	^{127}I (n, γ) ^{128}I	24	.99	m	442	.9
Cd	PT [b]	K	^{41}K (n, γ) ^{42}K	12	.36	h	1524	.7
Cd	PT [b]	Mg	^{26}Mg (n, γ) ^{27}Mg	9	.46	m	843	.8
Cd	PT [b]	Mn	^{55}Mn (n, γ) ^{56}Mn	2	.58	h	846	.6
Cd	PT [b]	Na	^{23}Na (n, γ) ^{24}Na	15	.0	h	1368	.6
BPE	VT [c]	Sb	^{121}Sb (n, γ) ^{122}Sb	2	.70	d	564	.1
BPE	VT [c]	Sm	^{152}Sm (n, γ) ^{153}Sm	46	.7	d	103	.2
INAA								
None	VT [d]	Cr	^{50}Cr (n, γ) ^{51}Cr	27	.8	d	320	.0
None	VT [d]	Fe	^{58}Fe (n, γ) ^{59}Fe	44	.6	d	1099	.2
None	VT [d]	La	^{139}La (n, γ) ^{140}La	40	.22	h	1596	.5
None	VT [d]	Rb	^{85}Rb (n, γ) ^{86}Rb	18	.7	d	1076	.6
None	VT [d]	Sc	^{45}Sc (n, γ) ^{46}Sc	83	.8	d	889	.2
None	VT [d]	Se	^{74}Se (n, γ) ^{75}Se	120	.4	d	280	.0
None	PT [d]	V	^{51}V (n, γ) ^{52}V	3	.75	m	1434	.1
None	VT [d]	Zn	^{64}Zn (n, γ) ^{65}Zn	244	.4	d	1115	.5
None		Ni	^{64}Ni (n, γ) ^{65}Ni	2	.52	h	1481	.8

[a] m, minutes; h, hours; d, days

[b] Epithermal neutron flux 1.4×10^{11} n cm^{-2}s^{-1}; Irradiation time: 10 m ; decay time: 10 m ; Counting time: 5 m.

[c] Epithermal neutron flux 1.1×10^{11} n cm^{-2}s^{-1} ; Irradiation time: 24 h ; decay time: 1-2 d ; Counting time: 20 m.

[d] Neutron flux 2×10^{12} n cm^{-2}s^{-1} ; Irradiation time: 24 h ; decay time: 30-45 d ; Counting time: 2 h

4. Results and Discussion

4.1. Elemental Concentrations in Taiwanese AK

The elemental concentrations using ENAA and INAA techniques of freeze-dried AK taken from five-planted areas, including as tea bags and capsules. The prime sources of systematic error arose in this figure mainly from counting geometries. These were minimized through experimental design by ensuring identical conditions for both standard and samples (Chen, 2009; Ibrahim, 1994).

Nearly twenty elements were presented in the analyzed samples at widely differing concentrations, ranging from 10^{-4} to 10^{-2} μg/g among the various areas. The TARI has been cultivating and propagating this health food over 25 years (Chen, 2004; Chen, 2003; Chen, 1995; Liu et al., 1992). Nineteen elements have been quantitatively analyzed herein based on the γ-ray spectra, and among these elements only selenium is present in roots and fresh leaves and stems. Analysis of tea bags and capsules from five markets showed that concentrations of over half of the analyzed elements clearly differed among markets as illustrated in Fig 2 (a) and (b). This investigation hypothesizes that tea bags and capsules are not only originated from various farms, but are also made using different portions of AK, processing methods and recipes in Taiwan. The elemental contents of the root, stems and leaves taken from various farms and markets may be significantly affected by the characteristics of growing environment and soil, including soil-pH, soil type, and the physical-chemical form of soil elements and fertilizers. Therefore, estimating the fluctuations of elemental concentrations of each portion of herb and of its end-products is very challenging.

4.2. Root

Table 2 listed the elemental concentrations of roots of five planted areas of AK. The elemental concentration of potassium is higher in the roots than elsewhere ranging from (0.85±0.08)% to (1.43±0.02)%. Additionally, the elemental concentration of aluminum in Chuchi was found to be higher than at the other farms. The elemental concentrations of arsenic range from (0.27±0.07) to (0.51±0.01)μg/g among different farms. Arsenic can produce developmental toxicity, including deformities, death, and growth retardation in hamsters, mice, rats and rabbits. Specifically, the differences of Al, Br, Cl, Cr, Fe, K, La, Mn, Na, Sb, Sc, V and Zn at Chuchi, Al, As, Br, Cl, Cr, Fe, K, Mn, Na, Sb, Sc, V and Zn at Lalashan, Al, As, Br, Cl, Cr, Fe, K, La, Mn, Na, Sc, V, and Zn at Minder, Al, As, Br, Cl, Cr, Fe, K, La, Mn, Na, Sb, Sc, V and Zn at Puli, are identified. Meanwhile, not all of the elements displayed any consistency in their concentration in the five growing regions, indicating that a significant range exists in elemental concentrations for various growing farms (Chen, 2002). Rare earth elements, lanthanum and samarium, were also found to be most highly concentrated in roots, with concentrations ranging from 1 down to 10^{-2} μg/g.

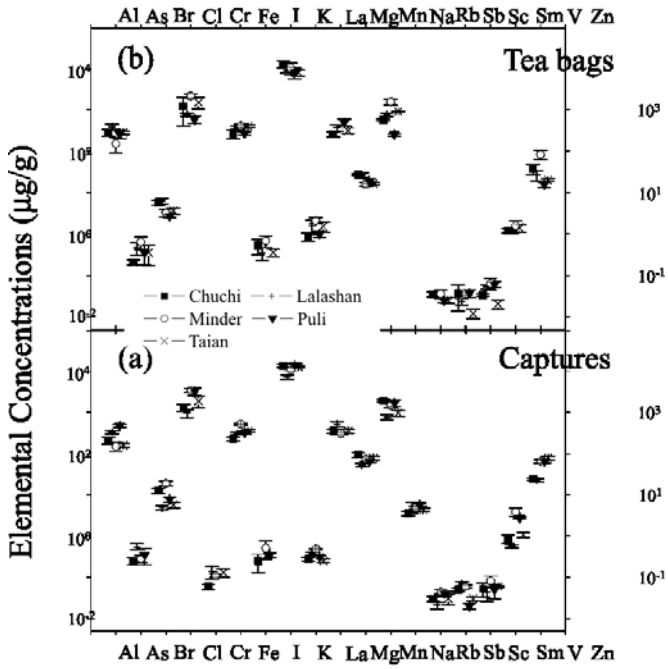

Figure 2(a,b). Elemental concentrations of capsules and tea bags distributed in the five planted areas.

Table 2. Elemental concentrations of roots (units μg/g or as otherwise stated, dry weight) for five planted areas of *Angelica keiskei*

Element	Planted area				
	Chuchi	Lalashan	Minder	Puli	Taian
Al	117±80	55±9	103±8	230±10	77±8
As	<MDC	0.42±0.02	0.51±0.01	0.27±0.07	0.46±0.05
Br	3.93±0.08	4.92±0.05	3.06±0.08	20.7±0.7	0.46±0.05
Cl(%)	0.18±0.01	0.26±0.01	0.12±0.01	0.07±0.01	0.09±0.01
Cr	<MDC	0.08±0.03	0.24±0.02	0.07±0.01	0.14±0.02
Fe	181±7	545±8	808±12	153±45	380±39
I	<MDC	<MDC	<MDC	<MDC	<MDC
K(%)	1.10±0.70	0.90±0.20	1.16±0.08	1.43±0.20	0.85±0.08
La	0.95±0.16	1.63±0.55	1.96±0.29	0.98±0.20	0.98±0.17
Mg	<MDC	<MDC	230±80	<MDC	190±30
Mn	36.3±1.3	17.6±1.0	58.0±2.1	37.4±5.6	34.4±3.8
Na(%)	0.054±0.003	0.128±0.003	0.080±0.005	0.104±0.014	0.068±0.010
Rb	1.30±0.40	<MDC	2.10±0.50	1.90±0.40	<MDC
Sb	0.030±0.008	0.095±0.002	0.051±0.012	0.048±0.010	0.032±0.007
Sc	0.030±0.005	0.188±0.016	0.266±0.023	0.045±0.005	0.223±0.013
Se	<MDC	0.25±0.10	<MDC	0.18±0.06	<MDC
Sm	0.125±0.057	0.150±0.051	0.229±0.053	0.184±0.037	0.181±0.049
V	1.10±0.23	0.88±0.19	1.51±0.44	0.17±0.03	1.24±0.20
Zn	27.8±3.8	39.2±6.0	12.1±2.5	60.1±4.3	22.6±4.9

4.3. Fresh Leaves and Stems

Nineteen elements were present in the analyzed samples at widely differing concentrations, ranging from 10^4 to 10^{-2} μg/g among the various planted areas. Therefore, classifying these elements revealed strong consistency among the concentrations of the trace elements which is interesting and is discussed below. Selenium is an essential element required in small amounts by humans for basics of life. It ranges from the highest 0.30±0.07 to the lowest 0.15±0.05 μg/g. In another example, the maximum concentration of As was 0.49±0.10 μg/g in Lalashan, whereas its concentration was negligible in both Minder and Puli.

Table 3. Elemental concentrations of fresh leaves and stems (units μg/g or as otherwise stated, dry weight) for five planted areas of *Angelica keiskei*

Element	Planted area				
	Chuchi	Lalashan	Minder	Puli	Taian
Al	136±10	126±19	107±21	129±29	165±20
As	0.28±0.07	0.49±0.10	<MDC	<MDC	0.33±0.07
Br	6.50±1.05	5.70±0.35	9.51±0.45	17.1±1.2	6.56±0.28
Cl(%)	0.23±0.03	0.35±0.07	0.17±0.03	0.09±0.04	0.51±0.23
Cr	0.10±0.03	0.19±0.01	<MDC	0.13±0.03	<MDC
Fe	300±68	360±40	310±80	380±140	280±70
I	0.37±0.07	0.35±0.07	0.82±0.17	0.67±0.19	0.30±0.04
K(%)	1.25±0.32	1.01±0.29	1.25±0.32	2.24±0.80	1.19±0.24
La	0.20±0.05	0.18±0.02	0.12±0.02	0.09±0.02	0.19±0.07
Mg	<MDC	<MDC	250±90	<MDC	130±10
Mn	50±6	83±25	37±7	63±10	33±12
Na(%)	0.069±0.006	0.140±0.044	0.075±0.004	0.168±0.016	0.163±0.045
Rb	1.50±0.36	2.21±0.43	<MDC	4.20±0.10	3.50±0.07
Sb	0.039±0.003	0.035±0.004	0.046±0.007	0.025±0.001	0.026±0.005
Sc	0.012±0.001	0.013±0.001	0.014±0.001	0.021±0.014	0.038±0.013
Se	0.20±0.06	0.30±0.07	<MDC	0.18±0.04	0.15±0.05
Sm	0.018±0.005	0.030±0.007	0.011±0.008	0.014±0.003	0.016±0.010
V	0.73±0.20	0.33±0.06	0.64±0.09	0.48±0.04	0.42±0.03
Zn	44±3	40±3	28±9	67±12	33±10

4.4. Capsules

Generally, capsules were made of old roots and tea bags were made of old stems and leaves (Yunming tea company, 1999; Liu et al., 1992). The potassium concentration is the highest both in capsules and tea bags elsewhere ranging from (1.35±0.15) to (0.74±0.09) percent. Magnesium and iodine were the first elements to be determined down to 0.1μg/g in the capsules. Mertz demonstrated that iodine is the active component of the thyroid hormones

triiodothyronine and thyroxin (Mertz, 1981). Among these elements, only Rb is present in capsules as shown in Fig 2b. Otherwise Rb concentrations in tea bags as well as fresh leaves and stems, sampled at different planted area can not be determined owing to having low concentrations. Concentrations of the elements Al, Cl, K and Na in the capsules generally differed significantly among the five markets, and only samarium displays consistently similar concentrations. Significant differences of Al, Br, Cl, Cr, Fe, K, La, Mn, Na, Sb, Sc, V and Zn at Chuchi, Al, Br, Cl, Cr, Fe, K, La, Mn, Rb, Sb, V and Zn at Lalashan, Al, Br, Cl, Cr, Fe, K, Mn, Na, Rb, Sb, V and Zn Minder, and Al, Cl, Fe, K, Na, Sb, V and Zn at Puli have been obtained. Meanwhile, the elemental concentrations of Fe, Mn, and Zn at Taian were revealed to be higher than at other markets, as listed in Fig 2(a). On the other hand, the concentrations of chloride at Minder and Puli were significantly higher than at other markets. An accurate determination of Se is important because Se deficiency directly causes Keshan and Kashi-Beck's diseases in low-Se areas (Chen, 2008).

4.5. Tea Bags

Meanwhile, arsenic was also found in the capsules and tea bags at levels of nearly 0.1 μg/g. Concentrations of iron and zinc were ranging from 500 to 20 μg/g. Additionally, the elemental concentrations of iron in capsules as well as zinc in tea bags in Minder were found to be the highest than at another planted area. Arsenic is classified as a toxic element while iron and zinc are essential for humans. It is indicated tea bags are not only made of the same portions of AK in five-planted areas but also being added with different recipes (Yunming tea company, 1999). The elemental contents in tea bags have shown good agreement with "drinking tea" of the public data from Wang (Wang et al., 1993), but zinc concentrations in tea bags of AK are higher than those in the drinking tea, especially, four times than that of Oolong tea (Wang et al., 1993).

Although the clinical recognition was limited by the scarcity of minor and trace elements, it is interesting to compare AK with three traditional Chinese herbs AS, LC and PG, all grown in humid climates. The comparative results indicate that the elemental concentrations in AK root generally resemble those in the roots/rhizomes of AS and LC, which have been used as Chinese medicine for more than four millennia, as listed in table 4 (Wang, et al., 1993). All of them belong to the same class: *Umbelliferae*. Furthermore, the concentrations of zinc in AK range from 12 to 60 μg/g, higher than the 14, 14, 19 μg/g of AS, LC and PG, respectively. Zinc is indispensable to life, and zinc deficiency has been linked to infertility, miscarriages, deformities, fetal intrauterine growth retardation, and premature and postmature births.

Furthermore, the concentration of iron in AK's roots ranges from 150 to 800 μg/g at different farms, exceeding the 120 μg/g in PG roots, as listed in table 4 (Wang et al., 1996).For instance, iron deficiency or anemia is proven to have a non-stochastic correlation with iodine deficiency whereas an excessive intake of bromine may cause health problems such as mental disturbance, dilated cardiomyopathy, uremia and lymphoma disease. The recommended nutrient density of selenium is not yet set. Most of the AS, LC and PG are still

collected from naturally grows plants in the jungle of mainland China. The contents of these elements of roots have been found to reflect natural level.

Table 4. Concentration range of some minor and trace elements in *Angelica keiskei* (AK), *Angelica sinensis* (AS), *Ligusticum chuanxioni* (LC) and *Panax ginseng* (PG)

Element (μg/g)	*Angelica keiskei* AK		*Angelica sinensis* AS[a]		*Ligusticum chuanxiony* LC[a]		*Panax ginseng* PG[a]	
Al	230	-1170	884		956		196	
As	0.27	-0.51	0	.66	0	.62	0	.34
Cl	730	-2590	1187		830		317	
Cr	0.072	-0.244	0	.03	0	.03	0	.150
Fe	153	-808	506	.5	510	.5	118	.6
K	8500	-11600	10740		9895		7891	
Mn	17.6	-58.0	18	.2	21	.3	23	.6
Na	540	-1280	161		938		72	.3
Sc	0.030	-0.095	0	.084	0	.092	0	.017
V	0.17	-1.51	0	.733	0	.851	0	.122
Zn	12.1	-60.1	13	.8	14	.4	18	.7

[a]Data taken from Wang et al., 1996.

4.6. Maximum Daily Intake of AK

It is important to investigate whether the toxic elemental contents in herbs or Chinese medicine may be considered critical from the human health point of view, especially for those populations consuming herbs in large quantities. Toxic elements such as As, and Sb are of prime interest in toxicological studies. From the perspective of bioavailability, only the simple ionic forms of elements can be absorbed by the human body, and thus it is interesting to estimate the MDI of metals from five AK farms in Taiwan. According to the habits of prescribing and consuming AK (Chen, 2002; Yunming, 1999), if an adult consumes 12 g/day, it would account for a MDI of 6.12 and 5.88 μg/g of arsenic in the roots and fresh stems and leaves, respectively (table 5). Assuming that three-year-old children have an average body weight of 15 kg, the MDI of arsenic concentrations is 3.06 and 2.94 μg/g in AK roots and fresh stems and leaves, well below the US recommended daily allowance for essential elements or Provisional Tolerable Daily Intakes (PTDI) values, and also below the 30 μg/g recommended by WHO/FAO (WHO, 1987). In these studies, trace element intakes could not be considerable harmful. A comparison with dietary reference intakes values (FAO/WHO, 2002) is shown in tab. 5. The contribution to the intake of Fe, Se, and Zn should be emphasized, as it represents an important contribution to the intake of these metals for the AK, particularly for the root, fresh stems and leaves, tea bags and capsules. Additionally, it is quite safe to consume these kinds of herbs. Concha et al. proved that arsenic was transferred to fetuses and suckling infants in a native Andean population, confirming that arsenic is easily transferred to the fetus during early human development and late gestation (Concha et

al., 1998). The concentrations of Cr, Fe, Mn and Zn are significantly less than the US recommended dietary allowances (RDA) for essential elements, and also less than the WHO/FAO provisional tolerable intakes (Food and Nutrition Board, 1989; WHO, 1989). Accordingly, the above indicates that the average Taiwanese probably exceeds the recommended daily intake of A1, Fe and Sc (Liu and Chung, 1991). On the other hand, the consumption of AK can provide numerous essential elements for consumers, such as Br, K, Mn, Na, Se and Zn.

Table 5. Maximum daily intakes (units µg/g or as otherwise stated) of elements from Taiwanese AK by adult and 15 kg body weight children

Element	root		Fresh stems and leaves		tea bag		capsule		RDI[a]	PTI[b]	Liu et al.,[c]
Al	2760		1980		4440		5640		-	-	9200
As	6	.12	5	.88	ND		ND		-	30	-
Br	248		205		84	.2	227		-	-	4200
Cl(%)	3	.12	2	.28	1	.90	4	.00	-	-	350
Cr	2	.88	2	.28	ND		1	.70	50	-	92
Fe	9700		4560		4850		4200		15000	-	8000
I	ND		9	.84	8	.16	6	.24	-	-	-
K(%)	16	.8	26	.9	11	.3	17	.0	-	-	170
La	23	.5	2	.40	4	.92	5	.88	-	-	-
Mg	2760		3000		3480		6480		-	-	-
Mn	696		996		259		1140		2000	-	2800
Na(%)	1	.54	2	.02	1	.40	2	.33	-	-	280
Rb	25	.2	50	.4	ND		67	.8	-	-	1700
Sb	1	.14	0	.55	ND		0	.50	-	-	-
Sc	3	.19	0	.46	0	.77	1	.30	-	-	0.2
Se	3	.00	3	.60	ND		ND		-	-	-
Sm	2	.75	0	.36	0.	94	1	.00	-	-	-
V	18	.1	8	.76	3	.36	46	.4	-	-	-
Zn	804		721		936		570		15000	-	7300

[b]Provisional tolerable intakes (WHO/FAO) for 15 kg body weight children.
[c]Analyzing daily dietary intake for citizen in Taiwan, see Liu et al., 1991.
[d]Non detected in this work.

The average (12 g/day for adult, 6g/day for children) ingestions, would account for a MDI of iron ranging from 4.2 to 9.7 mg/g, as listed in table 4. Dallman et al. proved that periods where iron intake is a particular concern include late infancy (6 months to 1 years, 10 mg/day), during which time the infant is increasingly dependent on dietary iron while their own iron stores have dwindled, adolescence, particularly during the growth spurt, and pregnancy, especially the latter half (Dallman and Simes, 1984). During the latter half of their pregnancy, the recommended iron intake for pregnant woman is 45 mg/day. Consumption of AK can provide large amounts of iron to infants and pregnant women (DOH, 1993). Specifically, Mn was identified as a constituent of mitochondrial glutamine systhetase, a

primary enzyme in the anti-oxidative defense system. The mean Mn of MDI determined by duplicate diet sampling and analysis by ENAA for the planted farms was AK root of Puli, 70 μg/day, and can provide large amount of Mn to adult and infant. As a result, arsenic was analyzed in roots and stems of AK. Greater attention should be paid to health effects on consumers. Besides arsenic, concentrations of Al, Br, Cl, Cr, Fe, K, La, Mn, Na, Sb, Sc, Se, Sm, V and Zn should also be determined in various portions of AK grown at different farms in Taiwan, thus obtaining information on the MDI of these elements in the human system through the herb consumed. Thus, the consumption of this herb could be of interest in the treatment of diuretic, analeptic, lactagogue. Further research is needed to ascertain the extent to which this herb has influenced the health of consumers in Taiwan.

5. Conclusions

This is the first study on elemental contents of AK among five farms in Taiwan, including both the winter and spring harvests. Up to 19 elements have been identified by ENAA and INAA using a triplicate portion technique. The results indicate a general agreement with AS and LC. The prescription (12 g/day for adult, 6g/day for children), the MDI of As is below that recommended by WHO/FAO, and thus the average daily intake of Al, Fe and Sc in Taiwan is probably excessive. Finally, the MDI of K, Cl, Na, Al, Br, Rb, V, La, Sm, Sc, and Sb correlate closely with the levels recommended by WHO/RDA. The levels consumed from these AK are safe and they are still recommended for providing children and adults readily of these health foods. The experimental results demonstrated that the INAA and ENAA approach can be applied successfully to analyze contents of AK. The levels consumed from these AK are safe, and they are still recommending for providing children and adults readily of these health food.

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Chapter 8

Pharmacologic Study of Some Plant Species from the Brazilian Northeast: Calotropis Procera, Agava Sisalana, Solanum Paludosum, Dioscorea Cayenensis and Crotalaria Retusa

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1. Introduction

The humankind needs to keep on exploring, in a rational manner, the chemical substances offered by living organisms, learning, copying and imitating the nature in its potential and structural diversity offered by laboratories of vegetal and animal analysis. Learning the chemical dynamism adopted by fauna and flora organisms will undoubtedly help the scientific progress of the nations. Besides, it will provide contribution for a better quality of life, protection and survival, comprehension and conservation of environmental conditions on planet Earth (Turolla, Nascimento, 2006; David, David, 2006).

The isolation and study of natural substances have been a central concern of chemical and biological sciences for more than 200 years. The Dictionary of Natural Products and its four supplements describe chemical, structural, pharmacological and bibliographic data of more than 100.000 natural products and related substances (Buckingham, 1993).

The alophatic medicine uses 119 drugs, which are extracted from about 90 species of superior plants. The existence of about 250.000 superior plant species allow us to deduce that many substances with medicinal activities can be isolated and structurally characterized from

these plants. The chemical potential of living organisms is of interest to pharmaceutical industries as a source of new drugs (e.g. taxol, ephedrine), agrochemical by providing natural fungicidal and insecticide (e.g. rotenon), food industries by providing natural substances for food flavor and color (e.g. menthol, benzoic acid), and cosmetics by natural perfumes (e.g. camphor, linalol, coumarin) (Buckingham, 1993; Braz-Filho, 2007).

According to Pereira (1993), there are about 12.000 known secondary metabolites, including terpenes, alkaloids, acetogenins, aromatics, and others used to intoxicate or dissuade insects or herbivore mammals of their predator action (e.g.: camphor, psoralen, florizin). Many of these insect-plant interactions cause clear evolutionary reflexes, which are responsible for different reactions that some plants present against their predators.

Medicinal plants are the ones that exert pharmacologic actions when administered to men or animals in any route or preparation (Brasil, 2001). They play important role in modern medicine, because they provide extremely important drugs that could hardly be obtained by chemical synthesis, as the alkaloids of *Papaver somniferum* and cardiotonic glycosides of *Digitalis spp* (Turolla, Nascimento, 2006). Although there is a clear development of huge pharmaceutical laboratories and synthetic drugs, medicinal plants remain as an alternative treatment in some parts of the world.

The World Health Association (WHO), through the Traditional Medicine Program, sponsors and encourages many countries to study the potential utility of traditional medicine, including the evaluation of efficacy and security of drugs derived from medicinal plants (WHO, 1993).

According to WHO estimates, a big part of the population, even in rich countries, still uses traditional medicaments, especially medicinal plants. Although it is easy to have access to modern medicine in these countries, the use of medicinal herbs remains popular for historic and cultural reasons. About 25% of all medical prescriptions have at least one product obtained from plants. On the other side, in developing countries, 65-80% of the population depends exclusively on medicinal plants for basic health care. This means that almost 4 billion of people trust in plants as sources of drugs (Agra *et al.*, 2007; Raven *et al.*, 2001).

There are about 500 thousand species of plants in the world. Brazil is one of the 12 nations that have 70% of the planet biodiversity and is the country with the biggest vegetal cover of the Earth. This fact has economic importance and also requires the conservation of such biodiversity (Brasil, 2001).

Nowadays, although the free official health system reaches the country, it cannot provide, in an adequate manner, the demand for health care. Furthermore, these people cannot afford health professional or industrialized drugs. Thus, they use medicinal plants, which are sometimes cultivated in their garden (Pilla, Amorozo, Furlan, 2006).

A great part of medicinal flora does not have its chemical and pharmacological properties well studied, and the popular knowledge about these plants exists predominantly in developing countries. The Brazilian Health Ministry has encouraged researches with traditional plants looking for possible new compounds and effects of these plants (Brasil, 2001).

In Brazil, especially in Northeast, about 80% of the population has low purchasing power and finds the cure for health problems in folk medicine, most of the time using plants

available next to their house or in little markets. This phytotherapy is called empiric phytotherapy (Matos, 2007).

In Brazilian Northeast, the people use about 700 species of plants for folk medicine. They self-medicate or are instructed by herb sellers, healers or rezadeiras. However, according to experimental data, especially pharmacologic, only 70 species are really medicinal and can be recommended for phytotherapic programs. This information does not mean that they do not have curative properties, but that the experiments cannot prove their effects (Matos, 2007).

Matos (1998) developed, in State of Ceará (Brazilian Northeast), a Project named Living Pharmacies, with the aim of giving assistance to communities which received few help from public health programs. These communities are instructed to use correctly plants with scientific proven actions (Table 1). This program encouraged studies on the uses and activities of these traditional plants and their products (Agra *et al.,* 2007; Quintans-Júnior *et al.,* 2002).

Many of the plants in Table 1 are widely used in Brazilian Northeast and have their effects proven by many studies. However, they need more specific chemical studies, in order to identify their active principles, and pharmacodynamic studies, to identify target for action, action mechanism and therapeutic doses.

At the Laboratory of Neuropsychopharmacology and Medicinal Plants of Ceará Federal University, our group studies the action of folk medicine plants of Northeast, such as *Calotropis procera*, *Agave sisalana, Solanum paludosum*, *Dioscorea cayenensis* and *Crotalaria retusa.* In this chapter, we discuss the pharmacologic and therapeutic activities of these species.

Table 1. Plants whose active principles are pharmacologically proven.

Popular Name	Species	Activity
Aroeira-da-praia	Shinus Terebinthifloius Raddi	Anti-inflammatory, heal mucosal wounds
Chambá	Justicia pectoralis var. stenohylla Leon	Anti-inflammatory, bronchodilator, anti-rheumatic
Courama	Kalanchoe brasiliensis	Local and systemic anti-inflammatory
Cumaru	Amburana cearensis	Bronchodilator, expectorant and systemic anti-inflammatory, anti-cholinesterase activity
Goiabeira-vermelha	Psidium guajava	Anti-diarrhea, antibiotic for Shigella, Salmonella and Serratia
Hortelã-rasteira	Mentha x villosa var.	Antibiotic for Amoeba, Giardia and Trichomonas
Malva-santa	Plectrantghus barbatus	Gastric hypo-secretor
Mavariço	Plectranthus amboinicus	Systemic anti-inflammatory
Melão-de-são-caetano	Momordica charantia	Anti-exoparasites, anti-larval for Ancylostoma
Mentrasto	Ageratum conyzoides	Local and systemic anti-inflammatory, local and systemic anti-rheumatic, antitumoral

Table 1. (Continued)

Popular Name	Species	Activity
Mororó	Bauhinia unguiculata	Anti-hyperglycemic, anti-lipidemic
Romã	Punica granatum	Antiviral for genital herpes
Torém	Cecropia pachistachia	Anti-hypertensive
Capim Santo	Cymbopogon citrates	Calming, sedative
	Auxemma glazioviana	Anti-cholinesterase activity

2. Calotropis Procera

Calotropis procera (Ait.) R. Br. (Figure 1), of the family Asclepiadaceae, comes from Africa, India and Persia. It is popular known as "ciúme", "ciumeira" or "algodão de seda", "flor-de-seda", "leiteiro" and "queimadeira". The scientific name of the family is derived from Asclepius, Greek medicine god, while the name of the species has its origin from the Greek "kalos" = beautiful, "tropis" = ship and *procera* from Latin "procerus" = high (Kissmann, Groth, 1999).

It is a perennial, shrub or subarborea plant, which can reach 3m height. Its branches, leaves, apples and fruits are covered by serosa, with high proportion of white latex, which flows abundantly when the tissues are broken (Joly, 1979; Rahman, Wilcock, 1991).

C. procera is a typical plant of Asia. It was brought to Brazil as an ornamental plant (Morcelle, Caffini, Priolo, 2004), which was rapidly spread through Brazilian Northeast due to the facility of dissemination of its seeds by the wind. The climatic condition of Brazilian Northeast is highly favorable to the growth of this plant. It is sometimes classified as invasive plant, because it can grow in places with adverse conditions, invading non-occupied niches. It was fast included in the group of plants that could be used in folk medicine.

The plant is famous by its great production of latex (Ramos *et al*., 2007). Latex is a vegetal liquid of milky texture that has many biologically active compounds, such as proteins, amino acids, carbohydrates, lipids, vitamins, alkaloids, carbonates, resins, tannins and terpenes (Morcelle, Caffini, Priolo, 2004). It may be obtained by a trivial process based on centrifugation and dialysis. The main constituent is the rubber, which is highly insoluble in water (Ramos *et al*., 2006).

When the plant suffers a mechanic injury, its tissues are broken and secrete latex. In contact with the air, latex coagulates and yields rubber. The characterization of the compounds of latex reinforces the idea that latex plays a role in defense mechanisms against virus, fungi and insects. This natural secretion has adhesive action and may immobilize insects (Moursy, 1997).

Glycosilated flavonoids extracted from the leaves, such as isorhamnetin-3-O-rutinoside and isorhamnetin-3-O-robinobioside, were identified by nuclear magnetic resonance techniques. The presence of organic carbonates was also assessed (Gallegos-Olea *et al*., 2006). Other chemical substances were identified, such as alkaloids, anthocyanins, proteolytic enzymes, cardenolids, cardioactive glycosides and triterpenes (Kumar, Basu,

1994; Melo *et al.*, 2001). Other flavonoids such as quercetins, resins, saponnins and tannins were also found (Salunk *et al*., 2005).

Source: comons.wikimedia.org/wiki/File:calotropis_procera.jpg

Figure 1. Photo of the species Calotropis procera

Different parts of *C. procera* have been used as phytotherapics to treat many diseases in traditional Indian medicine. They have also been used as analgesics, anti-inflammatory, purgatives, anti-helminthic, larvicide, nematocide, anticancer, in the treatment of gastric ulcers, hepatic disease and as antidote for serpent poisoning (Aktar *et al.* 1992; Basu, Chaudhuri, 1991). However, Sharma *et al.* (1934) describe that latex may be very irritant and corrosive and is used with criminal intention (abortive and infanticide).

2.1. Pro-inflammatory, Anti-inflammatory and Allergenic Properties

C. procera latex is well known for its medicinal and toxic properties. When locally administered, latex induces an intense inflammatory process that can be characterized by increase in vascular permeability, edema and cellular infiltrate (Padhy, Kumar, 2005). Such inflammation is due to histamine derived from mast cells and also to the histamine present in the latex. Thus, anti-histamine drugs can be used in the treatment of inflammation caused by the exposition to the latex of this plant (Shivkar, Kumar, 2003).

The inflammation caused by latex has been demonstrated by many experimental models of inflammation, such as paw edema, air bag (Singh *et al.*, 2000) and rat pleurisies (Shivkar, Kumar, 2003). Thus, latex is a potent flogistic agent that may be useful as a tool for research of new anti-inflammatory drugs.

The inflammatory effects caused by proteins present in dried latex can be effectively inhibited by cyproheptadine and refecoxib. The latter is superior to diclofenac in ameliorating the hyperalgesia induced by DL (Sehgal, Kumar, 2005).

However, latex has also anti-inflammatory effects. Oral administration of aqueous methanolic latex extract inhibited edema induced by carragenin and formalin. Histological analysis demonstrated that latex extract was more potent than phenylbutazone to inhibit the cellular infiltrate and subcutaneous edema induced by carragenin, suggesting that latex inhibits mainly histamine and bradykinin (Arya, Kumar, 2005).

Latex inhibited inflammation in models of paw edema and air bag in rat experiments (Kumar, Basu, 1994). Treatment with methanolic extract inhibits articular inflammation, probably due to reduction of cellular influx and vascular permeability. These results suggest an anti-arthritis activity of latex (Kumar, Roy, 2007).

Latex proteins may cause allergenic responses when in contact with the skin of susceptible people. These proteins are sequestered during latex coagulation (Ramos *et al.*, 2007). Some proteins of this latex show a basic Isoelectric Point (IP) with molecular weight (MW) from 5 to 95 kDa. The most common protein has MW of 26 kDa. Among the proteins, cysteinyl proteases, quitinases and antioxidant proteins (superoxide dismutase) are found (Freitas *et al.*, 2007). Assays showed that resolubilized proteins of coagulated latex and non-treated latex were able to stimulate immunologic activity when administered subcutaneously, developing cutaneous anaphylaxis (Ramos *et al.*, 2006).

Non dialyzed fraction of latex (NDFL) shows anti-inflammatory and analgesic activity when intraperitoneally administrated. It would be interesting to assess if NDFL has these activities by oral administration, because it does not produce allergy by this route (Ramos *et al.*, 2007).

2.2. Antinociceptive Properties

Latex powder by oral administration decreased significantly abdominal contortions induced by acetic acid. Such effect was stronger when compared to pre-treatment with aspirin (Dewan, Sangraula, Kumar, 2000).

The anti-nociceptive activity of latex protein was proven by many pharmacological tests of nociception using mice, such as acetic acid induced contortions, formalin test and hot plaque test. Such effect justifies the use of this plant in folk medicine and may be independent on opioid system (Soares *et al*., 2005).

Studies have shown that chloroformic fraction of *C. procera* roots has potent anti-inflammatory activity against exudative and proliferative stages of inflammation. Besides, this fraction also showed analgesic effects in acetic acid induced alterations in rats (Basu, Chaudhuri, 1991).

2.3. Antitumoral Properties

Antitumoral activity of latex was tested in assays using different lines of tumor cells, and it was able to inhibit the growth of the cells lines HL-60 (leukemia), HCT-8 (cervix cancer), MDA-MB-435 (breast cancer) and F295 (brain cells cancer) when analyzed by MIT (Oliveira *et al*., 2007).

The citotoxic activity of the laticifer protein (LP) of latex was also tested *in vitro*. LP showed citotoxicity IC (50) values of 0.42 and 1.36 μg/ml to cell line SF295 and MDA-MB-435, respectively. Effects on the cell viability and morphology were not seen in healthy mononuclear cells of peripheral blood exposed to LP (10μg/ml) for 72h (Oliveira *et al*., 2007).

2.4. Hepatoprotector Properties

Liver is an organ of vital importance to the metabolism of exogenous and endogenous challenges like drugs, viral infections, xenobiotics and chronic alcoholism. Thus, if the natural defense mechanisms are not efficient to eliminate these metabolites, hepatocytes will suffer injuries (Setty *et al.,* 2007).

In the model of hepatotoxicity induced by carbon tetrachloride (CCl_4) in rats, latex showed potent antioxidant and hepatoprotector properties. Histological analysis of rat liver demonstrated necroinflammatory changes associated to increase in the levels of thiobarbituric acid (TBARS), PGE_2 and catalase, and reduction in the level of glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GPx). Antioxidant and anti-inflammatory effects of latex and silymarin are similar, suggesting that latex can be used as hepatoprotector agent (Padhy, Srivastava, Kumar, 2007).

Hepatoprotector effect of *C. procera* extract was also observed in rats with acetaminophen induced hepatotoxicity. Alcoholic extract reduced the levels of hepatic enzymes TGO, TGP, alkaline phosphatase and bilirrubin (Setty *et al*., 2007).

2.5. Anti-diarrhea Properties

Studies have shown that the spasmogenic and carminative properties of this plant is due to its capability to contract smooth muscle of gastrointestinal tract (Sharma, 1934).

Latex powder decreased the frequency of diarrhea induced by castor oil (*Ricinus communis* L.), suggesting a possible anti-diarrhea activity similar to other drugs, like atropine and non-steroidal anti-inflammatory drugs, such as phenylbutazone (Kumar, Shivkar, 2004).

2.6. Larvicide Property

Aedes aegypti Linn is the vector of some endemic diseases like dengue and yellow fever. It is found in Africa and Latin America, mainly in Brazilian Northeast (Ramos *et al.*, 2006). Many natural products have been suggested as a way of chemical control of these mosquitoes.

Study performed in the 80's showed that *C. procera* latex has larvicide activity (Girdhar *et al*., 1984). It seems that latex is toxic to the eclosion of the eggs and to the larva of *A. aegypti*. Such property raises the possibility of a new formula to be used in Programs of Prevention and Control of *A. aegypti* and others mosquitoes vectors of diseases (Ramos *et al.*, 2006).

2.7. Other Important Properties of *C. Procera*

Alzheimer disease is an organic, chronic and progressive brain disorder, characterized by multiple cortical dysfunctions, including memory, comprehension, speaking and learning. Alzheimer disease does not have cure, but pharmacologic treatments can reduce the symptoms and slow the evolution of the disease. Studies suggest that latex can be used to treat of early symptoms of Alzheimer disease. Powder of latex reduced the deposition of β-amyloid in mice brain, suggesting a protective and antioxidant activity of this organ (Joshi *et al*., 2008).

The spasmolitic effect of aqueous extract of *C. procera* was assessed *in vitro* by experiments using smooth muscle of tracheas of pig. The extract, in doses 50, 100 and 200μg/ml, showed dose dependent relaxant activity of smooth muscle (Iwalewa, Elujoba, Bankole, 2005).

Ethanolic and aqueous extracts of *C. procera* roots showed contraceptive effects in albino female rats. They interfered with estral cycle and inhibited ovulation (Circosa, Sanogo, Occhiuto, 2001). A strong anti-implant (100% of inhibition) and uterotropic activities were seen at dose 250mg/Kg (1/4 of LD_{50}). No anti-estrogen activity could be detected (Kamath, Rana, 2002).

Ingestion of dry and chopped leaves of *C. procera* by goats, at concentration of 60% in diet, during 40 days, does not produce clinical or serum enzymatic alterations (Melo *et al*., 2001). Thus, *C. procera* can be used as a complement of animal rations during dry seasons. It

represents 16.7% of rations offered to confined animals and does not alter the taste and texture of the meat (Madruga *et al.*, 2008).

3. Agave Sisalana

Agavaceae family consists of about 25 genus and 650 species and is distributed predominantly in Pantropical regions, especially in the arid ones. In Brazil, four genuses and about 20 species are found. Sisal (*Agave sisalana*) is a plant which produces fiber and whose culture is an important alternative to the dry areas of Brazilian Northeast (Souza, Lorenzi, 2005).

The plant has long and hard leaves that come from a short trunk. The trunk stays in the soil, like a rosette. When old, it emits a big inflorescence, but it does not fructify (Rizzini, Mors, 1976).

A. sisalana has economic importance to Brazilian Northeast due to the production of hard fibers. However, during grinder, a vegetal residue known as "suco de sisal" (sisal juice) can be obtained. It has been used, mainly by other countries, as raw material to produce medicaments (mainly hormones), insecticides, proteic and vitaminic complexes for food and rations (Pimentel *et al.*, 2008).

The main uses of sisal are the production of rope, thick string, carpet, etc. The leaves can also be used to produce cellulosic paste for paper (Rizzini, Mors, 1976).

The filtrated juice of leaves has inhibitory activity against the growth of fungi, like *Aspergillus flavus* and *A. parasiticus* (Pires, Purchio, 1991). It is also possible to find steroidal sapogenins in this juice. Among the sapogenins, hecogenin is the most important to the synthesis of corticoids, in which hecogenin is the raw material (Rizzini, Mors, 1976).

4. Solanum Paludosum

Solanaceae family has a cosmopolitan distribution, mainly in Neotropical region, and comprises more than 150 genus and 3000 species. In Brazil, 32 genus and 350 species are found *(Souza e Lorenzi, 2005). Plants of Solanum genus produce a great variety of steroids, saponins and glycoalkaloids, which make the plant resistant to many pests* (Silva *et al.*, 2005).

Solanum paludosum Moric. is a neotropical species that is widely spread in South America, especially in Venezuela (Bolivar State), Guiana, Suriname, French Guiana and Brazil. In the latter, it can be found from North region to Rio de Janeiro. In Northeast of Brazil, *S. paludosum* is popularly known as "jurubeba-roxa", whose fruits are considered toxics (Basílio, Agra, Bhattacharyya, 2007).

Many chemical substances were isolated from *S. paludosum*, like steroidal alkaloid, solasodine and other compounds: triterpenos, glycosilated steroids and flavonoids (Basílio, Agra, Bhattacharyya, 2007; Schenkel, Gosmann, Athayde, 2007). A chemical study of seven species of *Solanum* showed that *Solanum paludosum* moric. produces two triterpenes, acetate of beta-amirine and beta-amirine, an alcamide (N-para-coumaroiltiramina), protocatecuic

acid, two steroidal saponins (3-O-beta-D-beta-glicopiranosilsitosterol and 3-O-beta-D-beta-glicopiranosilestigmasterol) and eight flavonoids: 3,4',7,8-tetra-O-methylgossipetine, 3,3',4',7,8-penta-O-methilgossipetine, 3,3',4',7 tetra-O-methylquercetine, 3-O-methyl-quercetine, 7-O-methylapigenine, 3,7-di-O-methylkanpherol, 7-O-methylkanpherol and 3,7,8-tri-O-methylherbacetine (Silva, Carvalho, Braz-Filho, 2002).

It is a species of potential importance in pharmacology, due to curarizing activity of its roots extracts and molluscicidal activity of its fruits (Silva *et al.*, 2005).

A study performed with species of genus *Solanum* concluded that extracts from fruits of *S. asperum* (CL50 = 420,5 μg/mL) and *S. paludosum* (CL50 = 548,0 μg/mL); aereal parts of *S. diamantinense* (CL50 = 481,0 μg/mL) and *S. sisymbrifolium* (CL50 = 382,7 μg/mL); and roots of *S. asperum* (CL50 =593,4 μg/mL) and *S. stipulaceum* (CL50 = 823,1 μg/mL) that showed molluscicidal activity against *Biomphalaria glabrata,* also showed toxic activity against *Artemia salina (*Silva *et al.*, 2007).

Other study assessed the anticonvulsant activity non-treated ethanolic extract of *S. paludosum.* In preliminary behavioral pharmacological trial tests, the extract showed depressor effect in central nervous system (Quintans-Júnior *et al*., 2002).

5. Dioscorea Cayenensis

Dioscoreaceae has predominantly Pantropical distribution and comprises five genus and about 900 species. Most of the species belong to *Dioscorea* (Souza, Lorenzi, 2005).

Dioscorea cayenensis produces the sapogenin diosgenin, whose commercial extraction is done almost always from *Dioscorea* species (Rizzini, Mors, 1976).

Diosgenin is the sapogenin of major economic importance. Sapogenins are important to production of steroidal hormones. Almost two-thirds of all steroidal hormones are produced from diosgenin extracted from tubercles of many species. Other sapogenins are hecogenin and solasodine, produced by *A. sisalana* and *S. paludosum*, respectively (Rizzini, Mors, 1976).

Solasodine belongs to the group of alkaloids because it presents nitrogen in ring “F”, while diosgenin and hecogenin have oxygen. The main differences in chemical structures of diosgenin and hecogenin are: hecogenin has carbonyl linked to carbon 12, while diosgenin has double bond between carbons 5 and 6 (Barbosa-Filho, 1991).

6. Crotalaria Genus

Plants of genus *Crotalaria* belong to family Leguminosae, which comprises more than 600 species and grows abundantly in tropical and subtropical zones. They are more numerous in Africa, India, Mexico and Brazil, which are the main centers of diversity of this genus (Cheecke, 1988; Palomino, Vásquez, 1991). In Neotropical region, approximately 70 species are found from the South of United States to subtropical Argentina and Uruguay. The species of genus *Crotalaria* are herbal plants with 50 cm height or trees with 3 m height, with digitate-trifolioate leaves, unifolioate or simple; predominantly yellow flowers; androecium

forming an opened tube at the base with dimorphic anthers. The species of this genus have great plasticity, being adaptable to different conditions. They may exist in many habitats, like areas next to rivers, sand dunes, restingas, edge of tropical forest, field and cerrado. The species are opportunists and occur in modified places, such as margins of roads (Flores, Miotto, 2005). The plants of *Crotalaria* genus are named “xique-xique”, “guizo de cascavel”, “chocalho de cascavel” and have dried pods that, when touched, emit a sound similar to that of rattlesnake tail (Williams, Molyneux, 1987).

In Brazil, more than 40 species were found. Many of these plants are food of animals, mainly in dry seasons (Hoehne, 1939; Tokarnia *et al.*, 2000). The most known toxic species are *Crotalaria spectabilis, C. crispata, C. retusa* (Figure 2), *C. dura* and *C. globifera* (Barri, Adam, 1981).

Source: Honório Júnior, J.E.R.

Figure 2. C. retusa.

The main use of the plants of *Crotalaria* genus is agriculture, by green adubation and vegetal cover of soil, which helps plantation. Plants of this genus are of great interest due to the intoxication and death of bovines. Other problem is the exposition of humans to these plants in folk medicine (Atal, Sawhney, 1973; Mattocks, 1986). Studies performed by our group showed that *C. retusa* has anxiolitic action at doses of 50 and 100mg in Swiss mice (Honório-Júnior *et al*., 2008).

6.1. Pirrolizidinic Alkaloid (PA)

These plants are rich in PA, which are the main plant derived toxins that affect humans and animals (Mattocks, 1986; Huxtable, 1990).

PA are found mainly in three plant families: Boraginaceae, Compositae and Leguminosae. Some of the most toxic plants of these families are showed in the Table 2.

Many cases of intoxication by *Crotalaria* were described in many countries, in horses (Gibbons *et al*., 1953, Gardiner *et al*., 1965; Arzt, Mount, 1999; Nobre *et al*., 2004a,b), cattle (Barri, Adam, 1981; Winter *et al*., 1990), pigs (Peckham *et al*., 1974; Mcgrath *et al.*, 1975), birds (Norton, O'Rourke, 1979; Alfonso *et al*., 1993) and goats (Barri, Adam, 1984; Nobre *et al*., 2005). *C. retusa* and *C. crispata* are responsible by the disease named *Kimberley horse disease* or *Walkabout disease* in Australia (Rose *et al*., 1957a,b; Gardiner *et al*., 1965).

Table: Important toxic plants that have PA.

Family	Species
Boraginaceae	*Amsinckia intermédia* *Borago officinalis* *Cynoglossum officinale* *Echium lycopsis* *Heliotropium europaem* *Symphytum officiuale*
Compositae	Senecio alpinus S. brasiliensis S. cinerária S. glabellus S. intergerrimus S. jacobaea S. logilobus S. riddelli S. vulgaris
Leguminosae	Crotalaria retusa C. spectabilis C. crispat C. dura C. globifera

Monocrotaline (MCT) (Figure 3) is the main PA found in plants of *Crotalaria* genus. Although it is a hepatotoxic alkaloid, pneumotoxic, nefrotoxic, cardiotoxic, teratogenic and carcinogenic effects were also related (Mattocks, 1986; Thomas *et al*., 1996; Ribeiro *et al*., 1993; Cheecke, 1998; Medeiros *et al*., 2000; Kosogof *et al.*, 2001).

The main alteration observed in lungs are edema and congestion, with consolidated areas in parenchyma, causing interstitial and arteriolar lesion, inflammation, hemorrhage and fibrosis (Gardiner *et al*., 1965; Newberne, *et al.*, 1974; Nobre *et al*., 1994; Baybutt, Molteni, 1999; Baybutt *et al*., 2002; Copple *et al*., 2002).

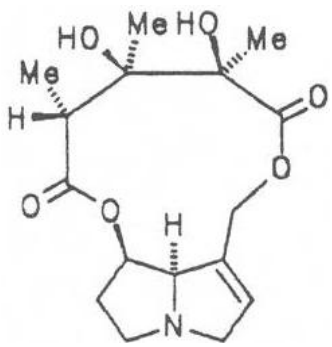

Figure 3. MCT molecule.

Schraufnagel (1990) studied lungs of rats treated with monocrotaline. He observed formation of new blood vessels, which occurs on pleural surface and on bronchovascular tree, but not in alveolar capillaries, suggesting that these capillaries answer in different ways to these angiogenic stimuli.

Deaths can occur due to damage on kidneys (Jubb *et al*., 1993). The main alterations are tubular damage and glomerulonephritis (Hayashi, Lalich, 1967; Carstens, Allen, 1970; McGrath *et al*., 1975; Figueredo *et al*., 1987).

Hepatocytes show citoplasmatic and nuclear gigantism (megalocytosis) (Bull, 1955; Cheeke, Shull, 1985; Mattocks, 1986; Thomson, 1990; Jubb *et al*., 1993). Other lesions are progressive fibrosis, bile ducts proliferation (Cheeke, Shull, 1985; Mattocks, 1986; Thomson, 1990) and occlusion of veins (Cheeke, Shull, 1985; Mattocks, 1986). There is impairment of metabolic liver functions. The levels of serum proteins are diminished due to the reduction in protein synthesis (Miranda *et al*., 1980), causing ascites and edema (Cheeke, Garman, 1974).

In liver, MCT is first activated to an electrophilic compound named monocrotaline pirrole (MCTP). It can also be named dehydromocrotaline (DHM), which have characteristics of a bifunctional agent of crossed linkage and has half-life period of 3s in aqueous with pH next to neutral. Stabilization of MCTP by red blood cells makes the transport to the liver easier. The evidences of the involvement of pulmonary endothelium in MCT intoxication is supported by the similarity between hepatic and pulmonary endothelium, evidence of thymidine increase (or deoxythymidine) and reduction of 5-hydroxitriptamine removed by endothelial cells and outflow of macromolecules. In primary pulmonary hypertension, disturbs on endothelial cell surface are suspects of being the initial factor to the formation of platelet aggregation and cause thrombosis *in situ* (Lamé *et al.*, 2000).

MCT is experimentally used to cause pulmonary vascular syndrome in rats, which is characterized by proliferative pulmonary vasculitis and pulmonary hypertension. MCT intoxication is also used as a model for studies in pulmonary hypertension (Lamé *et al.*, 2000). LD_{50} is 109mg/kg in male rats (Mattocks, 1972).

In vitro experiments using endothelial cells of bovine pulmonary arteries showed that MCTP reduced the capability of these cells to act as a permeable barrier and inhibited the cellular proliferation. Apoptosis occurs in endothelial cells of pulmonary arteries when MCT is *in vivo* administrated (Lamé *et al.*, 2000).

Many studies have supposed that MCT acts in endothelial cells to cause pulmonary hypertension. However, how these cells lose their functional capability is not known. MCTP seems to react easily with thiol group of cistein and glutathione.

Auto-radiography analyses showed that MCTP binds covalently to specific proteins. Lamé *et al* (2000) identified many proteins with targets for binding to MCTP, like: galectin-1, PDI precursor (protein-disulfide isomerase), probably protein disulfide isomerase ER-60, β- or γ-cytoplasmic actin and cytoskeletal tropomyosin. These proteins have functions which are potentially important to maintain the barrier of endothelial cells.

6.2. Chemical Structure of PA

Alkaloids are compounds that have nitrogen in a heterocyclic ring and generally have basic pH. They taste bitter, are physiologically and pharmacologically active and function as a chemical defense of plants against herbivores. PA are a big group of alkaloids that contains a pirrolizidine nucleus. They are widely spread, geographically and botanically. Many PA are hepatotoxic, causing irreversible damage to the liver, while others are carcinogens (McLean, 1970; Cheecke, Shull, 1985; Prakash *et al*., 1999; Cheeke, 1988).

These alkaloids become toxic after bioactivation and metabolism in liver to yield a dehydroalkaloid. These pyrrolic metabolites are potent alkylatins agents with short half-lives in aqueous. Dehydroalkaloids have four pathways available for further metabolism: hydrolysis to 6,7-dihydro-7-hydroxyl-1-hydroxymethyl-5H-pyrrolizide (DHP), alkylation of cell macromolecules, release into the circulation, or conjugation with GSH to form 7-glutathionyl-6,7-dihydro-1-hydroxy-methyl-5H-pyrrolizine (GSDHP). DHP and GSDHP have low toxicities compared to the parent dehydroalkaloid, so their formation can be considered to represent detoxification. The distribution of metabolites between these pathways determines both the sites at which toxicity is expressed and the degree of toxicity (Mattocks, Jukes, 1990).

Lipidic peroxidation was examined by Griffin and Segall (1987) as a possible mechanism of cell injuries caused by macrocyclic pyrrolizidine alkaloid of senecionine, a trans-4-OH-2-hexenal, isolated from rat hepatocytes. The results suggest that lipidic peroxidation that occurs in presence of trans-4-OH-2-hexenal is not totally responsible for cell damages in rat hepatocytes. Lamé and Segall (1986) showed that aldehyde dehydrogenase (ALDH) plays an important role in detoxication of trans-4-OH-2-hexenal, one of the metabolites of PA formed by lipidic peroxidation of membrane.

According to Cheeke (1988), most of hepatotoxic PA are necine esters base of retronecine and heliotridine, which are diastereomers with opposite configurations at C7. Their toxicity is influenced by structure. For hepatotoxicity, there must be a 1,2-double bond and a branch in an esterified side chain. Cyclic diesters are the most toxic, noncyclic diesters are of intermediate toxicity, and monoesters are the least toxic.

Pyrroles are very reactive and strong alkaline agents. They make crossed bonds with DNA and impair cell division and protein synthesis (Nobre *et al*., 2004a; Simões *et al*., 2004).

6.3. Toxicity: Experimental Data

Yan and Huxtable (1995) worked with MCT and trichodesmine, neuro and hepatotoxic alkaloids in rats. The authors observed that the level of glutathione (GSH) is increased more than 50% when MCT (65mg/Kg, i.p.) and trichodesmine (15 mg/Kg, i.p.) are administered. The ability of the rat to metabolize the two alkaloids was shown by the appearance of tissue-bound pyrrolic metabolites of pyrrolizidines in various organs. The levels of these metabolites appear to correlate with organ toxicity. For the hepatic and pneumotoxic alkaloid, MCT, higher levels are found in liver (17nmoles/g) and lung (10nmoles/g) than for trichodesmine (7nmoles/g and 8nmoles/g, respectively). For the neurotoxic alkaloid, trichodesmine, higher levels are found in brain (3,8nmoles/g tissue) than for MCT (1,7nmoles/g). Aziz *et al* (1997) showed that MCT increases oxidative cell stress.

According to Peckham *et al* (1974), 76 of 150 pigs died due to contamination of rations with 0.5% of *C. spectabilis* seeds. The first death occurred six weeks after start eating the rations and three weeks after the fall of the black hair of animal. The latter was the first signal observed. Other clinical symptoms were anemia, melena, edema and loss of weight. Blood samples were collected from 16 animals: six had symptoms of intoxication and ten looked healthy; one showed leucopenia and severe anemia; other animal had leucocytosis; three were lightly anemic; and eleven had normal hemogram. The following biochemical tests were performed with the serum of the ten healthy animals: urea, alkaline phosphatase, TGO (AST) and TGP (ALT). A slight increase in activity of alkaline phosphatase, TGO and TGP was observed. Deaths were observed following 16 weeks after start eating the rations, but most of the deaths occurred between 8^{th} and 12^{th} weeks. Toxic effects of the seeds were evidenced five months after its withdrawal of diet. The main pathological findings were gastric ulcers, hemoperitoneum, hepatic atrophy and fibrosis, renal fibrosis with glomerular cists and interstitial proliferative pneumonia. The microscopic injuries of great diagnostic value were hypertrophy and karyomegalia of hepatocytes and epithelial cells of renal tubules. The severity of the effects of intoxication correlated with the age of the pigs. The young ones were severely affected and the mortality rate was about 80%, while the group of old pigs (100 kg) showed a mortality rate of 8% (Nobre *et al*., 2005).

Horses intoxicated with *C. retusa* presented anorexia, dizziness, irritability, gape, muscular spasms, loss of coordination, head upside down, agressivity, to wander about and gallope aimless (Gardiner *et al*., 1965; Nobre *et al*., 2004a). Similar symptoms were described in intoxication of horses with other species of *Crotalaria* (Arzt, Mount, 1999;

Nobre *et al.*, 2004 a,b). Necropsy findings are typical of chronic liver disease. The liver is hard, increased in volume (Gibbons *et al.*, 1953; Gardiner *et al*., 1965; Nobre *et al*., 1994; Arzt, Mount, 1999) and of nutmeg looking (Arzt, Mount, 1999). In lungs, the main alterations observed were edema, congestion and consolidated areas of parenchyma (Gardiner *et al*., 1965; Nobre *et al*., 1994). Histologically, the mains findings occurred in liver: fibrosis, hepatomegalocytosis (Gibbons *et al*., 1953; Gardiner *et al*., 1965; Arzt, Mount, 1999), necrosis (Gardiner *et al*., 1965), vacuoles on hepatocytes (Nobre *et al*., 1994), hemorrhage (Arzt, Mount, 1999) and, sometimes, proliferation of biliar ducts (Gardiner *et al*., 1965; Arzt, Mount, 1999). The lungs may present diffuse fibrosing alveolitis with thickening of interalveolar septs, edema and mononuclear inflammatory infiltrate and, mainly, foam cells (Gardiner *et al*., 1965; Nobre *et al*., 1994). Horses exposed to metabolic and nutritional stress during dry season, hard working or pregnancy are more susceptible to be affected by the disease (Curran *et al.,* 1996). The chronic disease is the usual form of intoxication, and the clinical signs manifest weeks or months after eating the plant. The liver injuries are progressive and the death may occur months or years after ingestion of plant that contains PA (Cheeke, 1998).

Nobre *et al* (2005) reported an outbreak of acute intoxication by *C. retusa* of 80 sheeps. Anorexia, severe depression, mild jaundice, incoordination and recumbence were observed in 16 sheep that died within 12 h. The liver had a nutmeg appearance at necropsy and centrilobular necrosis was observed at histology. In order to reproduce the symptoms, seeds of *C. retusa* were given to six sheep at doses of 2.5 (two sheep), 5, 10, 20 and 40 g/kg (one sheep for each dose). Clinical signs and gross and histological lesions were similar to those observed in field outbreak. Nobre *et al* (2004b) observed the same symptoms in horses and asininos intoxicated with the seeds of *C. retusa*. The necropsy revealed type II Alzheimer astrocytes, isolated or in groups mainly in caudate nucleus and cortex.

Barreto *et al* (2006) demonstrated that MCT has a direct *in vitro* effects on astrocyte primary culture, interfering on cellular growth and inducing morphological changes, and suggests that the astrocytes' response to this alkaloid may be related to CNS damages and neurological signs sometimes showed by *Crotalaria* intoxication in animals. Low doses of dehydromonocrotaline show astrogliotic reaction. At higher concentrations (10-500 microM), astrocytes shrank their bodies and retracted their processes, presenting a more polygonal phenotype and a weaker expression on GFAP labelling nuclear chromatin, revealed condensed and fragmented chromatin in an important proportion (+/-30%), suggesting signs of apoptosis (Barreto *et al*., 2008). Signs of apoptosis were also observed at low and high doses of MCT in endothelial cells of pulmonary arteries (Thomas *et al.*, 1998).

Studies with cultures of endothelial pulmonary vascular cells of rats (RECs) show that MCTP caused a delayed and progressive release of lactate dehydrogenase from REC monolayers. Progressive cell detachment was evident and remaining cells became enlarged. Morphologic changes included cytoplasmic vacuolization, prominent stress fibers and nuclear enlargement. In addition to structural changes, MCTP inhibited cell proliferation at concentration of 0.05µg/ml. DNA crosslinking was evidenced at 24 and 48 hours post-treatment. Hoorn et al (1993) suggest, with this article, that MCTP is directly toxic to these cells. The cytostatic nature of the compound, in combination with its cytolytic effect on

RECs, could contribute to the development of pulmonary edema and other lung vascular changes seen in rats treated with MCT or MCTP.

MCT causes anxiolitic effects at acute doses of 50 and 100 mg/kg (i.p.) in Swiss mice. It lacks sedative activity (Honório-Júnior *et al*., 2008).

The exact mechanism of MCT toxicity is not known, but it is necessary that its biotransformation by liver yields the reactive metabolite DHM, which interferes in DNA and protein synthesis (Petry *et al*., 1984, Butler *et al.,* 1970).

6.4. Possible action Mechanism

According to Mattocks (1986), hepatotoxicity of PA is related to insaturation between carbons 1 and 2, which, in presence of oxidase, yield pyrroles. They bind to nucleophilic groups of macromolecules, as sulfhydryl, hydroxyl and amino groups of enzymes, globulin, hemoglobin, purine and pyrimidine. Thus, they make irreversible crosslinks with DNA and RNA and cause citotoxic, mutagenic and carcinogenic (Nobre *et al*., 2004a; Simões *et al*., 2004).

PA are activated *in vivo* to yield reactive pyrrolic intermediates that have been shown to cross-link DNA primarily (Kosogof *et al*., 2001; Tepe, Williams, 1999). Thus, MCT requires bioactivation to DHM (Figure 4) by P450 cytocrome in liver, a bifunctional reaction of an alkaline agent that binds to DNA *in vivo*. DHM seems to be neutralized by conjugation with glutathione (GSH). This way, amino acids like cistein, which contains sulfil and taurine, prevent the toxic effects of MCT (Yan, Huxtable, 1995).

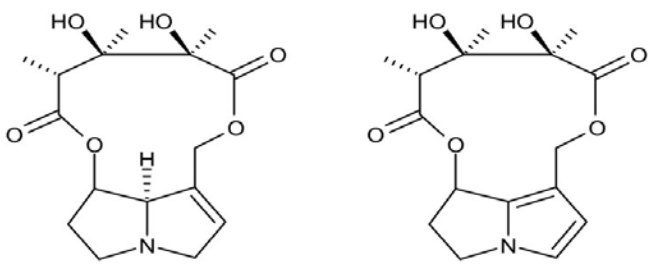

Figure 4. Structures of monocrotaline and dehydromonocrotaline.

Oxidation of substrate linked to energy reduces the equivalent products, which are transferred via NADH or $FADH_2$ in respiratory chain. An electrochemical proton gradient has the membrane potential as main component able to maintain ATP synthesis (Nicholls, 1982).

Such connection with mitochondria has been considered an important target to xenobiotics that can cause cell injuries via ATP depletion (Wallace, Starkov, 2000).

Tepe and Williams (1999) described the semisynthesis and DNA cross-linking of the first photochemically triggered progenitor of dehydromonocrotaline. A wide variety of pyrrolizidine alkaloids, such as monocrotaline, and the clinically significant mitomycins and the related FR-900482, FK 973, and FR-66979 exert their cytotoxicity through the formation

of DNA-DNA interstrand cross-links and DNA-protein cross-links. These naturally occurring antitumor antibiotics are generally either oxidatively or reductively activated in vivo forming a highly reactive pyrrolic-type intermediate, which is responsible for the ultimate DNA cross-linking reaction. Such oxidative or reductive paths can cause many toxic effects.

Photochemically triggered progenitor of a pyrrolizidine alkaloid generates dehydromonocrotaline upon photochemical activation. This oxidation results in the electrophilic activation of the C-7 and C-9 positions, via conjugation with the pyrrole nitrogen lone pair, which are prone to nucleophilic attack by DNA. The DNA crosslinking specificity of this reaction has been elucidated and demonstrated to occur at $^{5'}CpG^{3'}$ sites via the exocyclic amine of dG residues in the minor groove (Tepe, Williams 1999).

Kosogof *et al* (2001) demonstrated the viability that more structurally diverse masked DNA-reactive pyrrolizidine progenitors should be capable of being designed and synthesized for more selective oligonucleotide modifications. Such agents hold promise as useful tools to gain insight into the mechanism of DNA–DNA and DNA–protein cross-linking.

Pereira *et al.* (1998) showed that DHM alkylates guanines at the N7 position major groove of DNA, which has not been reported before. The reaction is rapid and there is preference for 5'-GC and 5'-GA sequences. DNA makes crossed bonds with DHM. Such aggregation (Figure 5) is reversible upon addition of EDTA or heat. These structures, apart from having radial appearance, are heat- and piperidine-resistant and are not reversible by any other chemical treatment. A more probable mechanism is the initial polymerization by DHM. Instead of forming a branched polymer, DHM could in fact form a dendrimer-like structure since it contains two eletrophilic sites and on nucleophilic site. Weak eletrophilic sites may have the potential to react with many fragments of DNA at N2 of guanines to form piperidine and heat-resistant DNA structures.

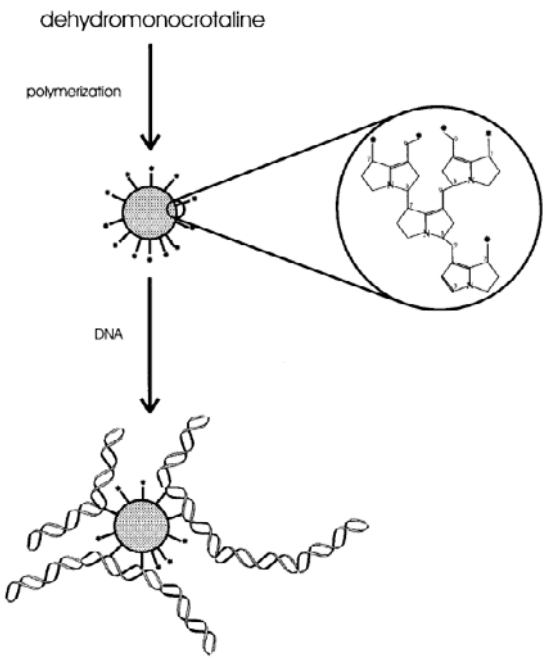

Figure 5. Schematic representation of polymerization of DHM and reaction of polymer with DNA.

DHM can modify DNA in different ways *in vitro*. It is possible that all of them may contribute to DHM-mediated DNA damage *in vivo*. DNA lesions in major groove are believed to be repaired by base excision repair, whereas minor groove lesions are repaired by nucleotide excision repair (Pereira *et al.*1998). DNA lesions induced by polycyclic aromatic hydrocarbons are repaired by both pathways, the contribution from each pathway depending on the extent of damage to guanine (Bralthwalte *et al.*, 1998).

7. Final Considerations

The results of these articles show that the plants presented here exert a diversity of effects and have potential for use as new drugs.

The pharmacological characteristics of *C. procera* should be explored by pharmaceutical industries for production of new antibacterial, anti-inflammatory and antitumoral drugs.

A. sisalana, S. paludosum and *D. cayenensis* are largely studied, but more knowledge must be acquired for a better use and, probably, discover of new therapies.

C. retusa is used in agriculture and MCT, its main toxic constituent, is used as pulmonary hypertension model in laboratory and induces oxidative stress. MCT makes DNA-DNA crosslinks that inhibits cell division. Many authors suggest a role of MCT in cancer treatment. New information is necessary to augment the knowledge of its effect in humans and its action mechanism and to discover new methods of detoxifying humans or animals that were intoxicated.

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Chapter 9

Ethnobotany and Experimental Pharmacology of *Echinodorus Grandiflorus* (chapéu de couro)

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Abstract

Echinodorus grandiflorus (Cham. & Schltdl.) Micheli and Echinodorus macrophyllus (Kunth) Micheli, are monocotyledonous species belonging to Alismataceae family. These plants are aquatic or semi-aquatic herbs, with submersed, floating or emersed leaves and with inflorescences that remain flourished during nearly 30 days. In Brazil, they are popularly known as "chapéu de couro" and have been used in the folk medicine in the treatment of several disorders. Its leaves are resources for very common teas, used as diuretic and anti-inflammatory, blood depurative, against arthritis and skin diseases, liver maladies and renal affections, as well as against amygdalitis, pharyngitis, stomatitis and gingivitis. Several researches have suggested promising results on medicinal activities of "chapéu de couro". Some of those activities were observed in vivo, such as diuretic, anti-inflammatory, hypotensive and antihypertensive, antimicrobial, decholesterolizing, immunosuppressive and vasodilator. In vitro activities were also confirmed, such as trypanocidal, leishmanicidal and antineoplastic. In this work it is presented the ethno and experimental pharmacology, regarding the researches accomplished so far, besides the botanical characterization, geographic distribution, macro and microscopic description, chemical constituents and toxicology.

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Keywords: Alismataceae, Echinodorus, chapéu de couro.

General Considerations

Echinodorus grandiflorus (Cham. and Schltdl.) Micheli belongs to the family Alismataceae which gathers aquatic or semi-aquatic herbaceous plants with leaves submerged, flotation or emergent that can be recognized by the production of latex, basal placentation and fruits of the type achene.

Each inflorescence stays flowery for approximately 30 days and it produces about 220 flowers, being stood out above the leaves during the whole spring. The flowers are scentless, actinomorphics, shallow and they possess white corolla with numerous stamens and yellow pistils, exposed. The anthesis of the flowers happens in the morning and each flower lasts about eight hours. Their morphologic type allows the access of several insects. The visits of 21 species of bees were verified and of six species of beetles, being the bees the main pollinators (HAYNES and NIELSEN, 1994, VIEIRA and LIMA, 1997). The aspect of the leaves and of the inflorescence with flowers in anthesis of *E. grandiflorus* can be observed in the illustrations 1 and 2.

Echinodorus spp. has unisexual flowers and cylindrical fruits containing glands among ribs. *E. grandiflorus* ssp. *grandiflorus* holds more than 21 stamens, leaves have pellucid markings as dots or lines, petioles are at least five times larger than the sepals, and bracts are larger than the petioles.

Figure 1. *Echinodorus grandiflorus*: general aspect of leaves.
Photo: PIMENTA, D.S.

Figure 2. *Echinodorus grandiflorus*: general aspect of inflorescence holding flowers in anthesis. Photo: PIMENTA, D.S.

E. grandiflorus is popularly known as "chá-mineiro", "erva-de-pântano", "erva-de-bugre", "congonha-do-brejo", "erva-do-brejo", "chá de campanha", and, most commonly, "chapéu de couro" (CORRÊA, 1984, CORRÊA JÚNIOR *et al*., 1994, MARTINS *et al*., 1994, LEITE, 1995).

Geographical Distribution

Alismataceae consists of 11 aquatic and semi-aquatic genera and about 75 species. *Echinodorus* and *Sagittaria* are among the largest genera in the family, each with 26 and 25 species, respectively. Other genera consist of less than 10 species each (TANAKA, 2000). The genus *Echinodorus* occurs in USA to Argentina and is restricted to the western hemisphere (BEVILAQUA *et al*., 2001). According to Joly (1991), *Echinodorus* has its center of dispersion in Tropical America.

Haynes and Nielsen (1994) recorded the following species: *E. grandiflorus* ssp. *grandiflorus* (center-west, southeast and south of Brazil, Paraguay, north of Argentina and Uruguay, flowering and fruiting from October to May), *E. grandiflorus* ssp. *aureus* (Cuba, Mexico, Central America, Colombia, Venezuela and Brazil, flowering and fruiting year round), *E. macrophyllus* ssp. *macrophyllus* (Guyana, western Brazil and Bolivia, flowering from October to April) and *E. macrophyllus* ssp. *scaber* (southern Nicaragua, Colombia, Venezuela, Guyana, Suriname, southern Bolivia, Paraguay and Brazil, flowering and fruiting year round).

Macroscopic Description

E. grandiflorus is an aquatic plant with rhizome and emerged, perennial leaves that bear petioles. Base of leaf cordate, lobe ovate and apex of leaf ranging from sharp pointed to acuminate. Margin of leaf entire, leaf blade dark green in color, about 20 to 40cm length vs 15 to 35cm basal width, wrinkled surface, rough, 11 to 13 salient veins in the abaxial surface. Petiole long, coriaceous, measuring up to 1,5m length (depending on the environment), longitudinally channeled and provided with longitudinal ridges. It is probably the coriaceous aspect of the leaf blade that has given the plant the popular name of "chapéu de couro". When present, inflorescences have taxonomic value and, in the case of *E. grandiflorus*, they are panicles, which may have a single or many branches, as well as either small basal interns or successive pseudoverticillate (HAYNES and NIELSEN, 1994).

Microscopic Description

Traverse cuts of the limbo to foliate they demonstrate the presence of mesophyll of the type dorsiventral, with a layer of parenchyma differentiated palisade and from six to eight layers of spongy parenchyma. Canals secretory of latex were observed in the petioles and in the main ribs as well as secretory cavities of latex for the whole limb (PIMENTA, 2002). The collenchyma tissue is restricted the medium rib, possessing in this species two to three layers (SCREMIN-DAYS *et al*., 2002). The anatomy of *E. grandiflorus* is clearly adaptated to the aquatic atmosphere and specifically of emergent aquatic species. Besides the numerous constituent cameras of aerenchimas, from the root cortex, going by the petiole and constituting part of the main ribs of the limb to foliate, the species presents numerous diaphragms delimiting those cameras internally (SCREMIN-DAYS, 2000).

In the secretory structures observed in leaves, the epithelium is simple, delimiting the channels or cavities and such structures don't get to characterize laticifers, that would be more complex structures of secretion and of deposition of latex. According to Bona *et al*. (2004), the petiole of *E. grandiflorus* presents epidermis uniseriate with cells containing walls periclinals external curves and thin cuticle. The fundamental tissue can be divided in three areas: a chlorophyllian parenchyma in the furrows of the petiole; a colorless parenchima in the projections of the furrow, containing grains of starch and monocrystals and the aerenchyma with cameras surrounded by 16 to 25 cells. Those air cavities are obliquely divided lightly by diaphragms oblique constituted by a layer of parenchyma cells, the ones which, interlinked, they form triangular small intercellular spaces. The vascular bunches are collateral, being located in the periphery of the circumference, without protochilema gap and disposed in arch in the aerenchima, with protochilema gap. Schizogenic laticiferous conducts are present in the parenchyma among the gaps of air and, in larger amount, about of the whole circumference of the petiole. Pimenta (2002) correlated the larger concentration of latex in the periphery of the petioles with its chemical characteristics and defense physicochemical. The latex has a fraction that is water soluble and another fraction that is water insoluble, this way, the insoluble fraction could be aiding to seal the epidermis against possible offenses, as well was precipitating in the small intracellular spaces of diaphragms

impeding the invasion of the air cavities of the aerenchyma for the water. The soluble fraction would be already related to the defense against herbivory in the submerged portion of the petioles.

Chemical Constituents

They were identified in the leaves of *E. grandiflorus:*

1) FATTY ACIDS: linolenic acid and dodecanoic acid (TANAKA, 2000); palmitic acid (PIMENTA, 2002).
2) TERPENOIDS
   Essential oils: linalool, dihydroedulan, trans-caryophyllene, *alpha* humulene, E-farnesene, *beta* selinene, *alpha* farnesene, *delta* cadinene, E-nerolidol, caryophyllene oxide, humulene epoxide, bisabolone, drimenol, neocembrene, echinoic acid, cembranoid, phytol (PIMENTA *et al*., 2006).
   Diterpenoids: echinodol (MANS and HARTMANNS, 1993), echinoic acid (TANAKA *et al*., 1997), phytol, hardwikiic acid, (-) 15-etoxicleroda 3 acid, 13-dien - 15,16-olide-18-óic, acid (-) - (16)-hidróxi-cleroda-3,13-dien-16,15-olide-18 - oic (COSTA *et al*., 1999, TANAKA, 2000), acid (-) -15 hidroxicleroda-3,13 - dien-16,15-olide-18-oic, acid (-) -cleroda-3,13(16),14-trien-18-oic (TANAKA, 2000), chapecoderins A, B and C (KOBAYASHI *et al*., 2000c), echinodolides A and B (SHIGEMORI *et al*., 2002), solidagolactona-I (PIMENTA, 2002).
3) STEROIDS: 24-etilcolest-4-en-3,6-dion; 3ß-the-ß-D-glicopiranosil sitosterol (TANAKA, 2000); campesterol, stigmasterol, sitosterol (PIMENTA, 2002).
4) FENOLIC ACIDS: acid caffeic, acid ferulic and acid isoferulic (PIMENTA, 2002).
5) FLAVONOIDS: isoorientin, swertisin and isovitexin (PIMENTA, 2002; SCHNITZLER *et al*., 2004), swertiajaponin, and isoorientin-7,3'-dimetil-ether (SCHNITZLER *et al*., 2004).
6) ALKALOIDS: echinofillins A, B, C, D, F (KOBAYASHI *et al*., 2000°; KOBAYASHI *et al.,* 2000b).
7) DERIVED OF THE TARTARIC ACID: acids caftaric, chicoric, cafeoil tartaric feruloil, 2-the-feruloil tartaric and tartaric di-feruloil (SCHNITZLER *et al*., 2004).

Ethnopharmacology

The parts of *E. grandiflorus* used for the treatment of several illnesses they are the leaves and the rhizomes. The use of the leaves is mentioned in FARMACOPÉIA DOS ESTADOS UNIDOS DO BRASIL (1926, 1959); in Coimbra (1994), Corrêa Júnior *et al*. (1994), Teske and Trentini (1995), Nogueira (2000), Almança and Carvalho (2003). The use of the rhizomes was mentioned by Corrêa (1984), Lorenzi and Matos (2002) and Dutra *et al*. (2006).

Actions diuretic and anti-inflammatory are described in FARMACOPÉIA DOS ESTADOS UNIDOS DO BRASIL (1926, 1959).

The tea of the leaves is used as diuretic, depurative, against syphilis, diseases of the skin, diseases of the liver, renal disorders, tonsillitis, pharyngitis, stomatitis, gingivitis and besides interrupting the progress of the arteriosclerosis. In the treatment of rheumatic gout and neuropathic pain compresses are applied and to combat the prostatitis seat bath it is recommended with the tea. The rhizomes are used in cataplasms for hernias (LORENZI & MATOS, 2002; DUTRA *et al*., 2006).

Experimental Pharmacology

Anti-Inflammatory and Antinociceptive Activities

The treatment of mice orally with the hexane, methanolic and aqueous extracts, obtained of dry leaves, inhibited the edema of paws (BRITO *et al*., 1999), in the same way that the methanolic extract of the rhizomes. This last one had the anti-inflammatory activity appraised for the pleurisy induced by carrageenin, being observed decrease of the leukocyte migration (DUTRA *et al*., 2006).

The aqueous extract of the leaves and the methanolic extract of the rhizomes of "chapéu de couro" inhibited the abdominal writhings induced by intraperitoneal administration of acetic acid in mice, evidencing antinociceptive and anti-inflammatory activity (CARDOSO *et al*., 2003).

Diuretic Activity

Diuretic activity was demonstrated, in female rats treated with the ethanolic extract (50%) of leaves of "chapéu de couro" (RIBEIRO *et al*., 1988) and in mice treated with the tea of the leaves, (CARDOSO *et al*., 2003), not being observed the same result for the ethanolic extract (80%), also obtained of leaves, and for the fraction ethyl acetate (COSENZA *et al*., 2008).

Hypotensive and Anti-hypertensive Activities

In mice naturally hypertensive, the hydroalcoholic extract of the leaves of "chapéu de couro" to 50% strongly reduced the blood pressure and also decreased the heart frequency (RIBEIRO *et al*., 1986). The administration in growing doses of the ethanolic crude extract, intraperitoneally, induced a dose-dependent anti-hypertensive effect, with fall of the blood pressure, of the heart debit and of the systemic vascular resistance, not having significant alteration in the heart frequency (ARAÚJO *et al*., 2001); similar results were observed by Lessa *et al*. (2008) with intraperitoneal, intravenous or chronic oral administration of the same extract, in the same experimental model, also showing reduction of the mean blood

pressure, which was parallel to the reduction of the cardiac debit and of the systemic vascular resistance.

In anesthetized normotensives animals, the aqueous and hexanic extracts of the leaves, administered intravenous, induced hypotensive effect reversible and dose-dependent (PIMENTA *et al*., 1998a). Similar results were found with the same extracts in hypertensive mice, when it was verified sharp inhibition of the synthesis of nitric oxide and significant reduction of the blood pressure (BARROS *et al*., 1999).

Polacchine (2005) evaluated the anti-hypertensive action using normotensive and hypertensive animals, treated intravenously with different doses of the aqueous crude extract, noticing reduction of the mean blood pressure and blockade of the action of the adrenaline.

Vasodilating Activity

In studies using as experimental model the isolated aorta of rabbits, relaxation of 60% was observed (ALMEIDA *et al*., 2000) to 65% (ALMEIDA *et al*., 2001), using the aqueous extract of leaves and of 81%, with the insoluble fraction in methanol, obtained starting from the extract aqueous liofilized (ALMEIDA, 2004).

Cailleaux *et al*. (2000), using the hexanic, methanolic, aqueous and ethanolic extracts of leaves, droughts or breezes, in isolated kidneys of rabbits, observed decrease, significant and dose-dependent, of the perfusion pressure, being the best result obtained for the aqueous extract. The aqueous extract seems to exercise its vasodilating activity through the mediation of activation of receptors of nitric oxide and PAF, not presenting signs of prostaglandins generation or activation of potassium channels, suggesting that the hypotensive effect of the extract would be due to a potent systemic vasodilator effect (TIBIRIÇA *et al*., 2006).

Antimicrobial Activity

The aqueous extract presented activity against *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhimurium* (DUARTE *et al*., 1999) and methanolic extract of the leaves against *Bacillus subtilis* and *Micrococcus luteus* (SOUZA *et al*., 2004).

In Vitro Trypanocidal Activity

Trypomastigote forms of *Trypanosoma cruzi* were incubated with the lyophilized aqueous extract, obtained of dry leaves, causing 90% of mortality after incubation for 24 hours (PIMENTA *et al*., 1998b). With the hexanic crude extract and with the partitions in butanol and ethyl acetate from the methanolic crude extract (GIBALDI *et al*., 2001), the mortality of metacyclic trypomastigote forms was 100%.

Leishmanicide Activity

The hexanic, methanolic and aqueous crude extracts, obtained of leaves, and the fractions hexane, dichloromethane, ethyl acetate, butanol and aqueous residue obtained of the partitions of the methanolic extract were evaluated against *Leishmania major*'s promastigotes, in infected mice. The more apolar constituents evidenced activity, being the more effective the hexane extract and the hexane and dichloromethane fractions (PIMENTA, 2002).

Hypocolesterolemic Activity

Cardoso *et al*. (2005) evaluated the activity of the aqueous extract on the plasma levels of cholesterol in mice treated with the oily solution of cholesterol in comparison with control animals and they verified the reduction of 2% of the cholesterol in treated animals with the tea to 5%, and in normal animals, for which the tea was given to 2,5%.

In Vitro Antineoplastic Activity

Pimenta (2002) conducted a screening for pharmacological assessment of the ability to inhibit proliferation of tumor cells through *in vitro* tests, using the extracts of cultures of strains of tumor cells SP2/0 (myeloma of mouse), NEURO 2A (neuroblastoma of mouse), J774 (macrophages of mouse), LLC-MK2 (epithelial cells from kidney of monkey), Erlich carcinoma (sarcoma induced by metilcolantreno), BW (the mouse lymphoma) and P3653 (plasmacytoma of mice). The crude extracts (hexanic, methanolic and aqueous) and the partitions more apolar from the methanolic crude extract (dichloromethane and hexane fractions) were the most promising against neoplasia.

Toxicity

Most of the studies on toxicity of *Echinodorus* refer to *E. macrophyllus* that is not object of that chapter. The toxicity of *E. grandiflorus* was not still studied appropriately, having only two works in the literature.

In 2005, Polacchine evaluated the acute toxicity in mice, treated with the tea of the leaves of *Echinodorus grandiflorus* orally in the doses of 0,5, 1,0, 2,0 and 4,0g/kg of body weight, no deaths were recorded in 24 hours.

Brugiolo *et al*. (2008) evaluated the effect of the doses of 500 and 1000mg/kg of the lyophilized aqueous extract of *Echinodorus grandiflorus* administered orally on the hematological and biochemical parameters of female Wistar rats, treated during 14 days post-insemination, observing anemia in the two doses and leukocytosis and hypercholesterolemia in the dose of 1000mg/kg.

Conclusion

Although many researches with the several types of extracts of *E. grandiflorus* have demonstrated promising results for several pathologies, future researches are necessary to be determined the safe use of this phytotherapic, mainly in clinical level. However, those extracts have being much used by the Brazilian population due to its diuretic and hypotensive activity.

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Chapter 10

Non-Invasive Near Infrared Spectroscopic Techniques for the Characterization of Medicinal Plants and their Constituents

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Abstract

Near-Infrared spectroscopy (NIRS; 800-2500 nm) is a non-invasive spectroscopic tool enabling a fast qualitative and quantitative characterization of medicinal plants and their constituents down to the ppm-level. Treatment of spectra recorded with chemometrical and multivariate approaches allows determining chemical (e.g. secondary plant metabolites, leading compounds) and physical parameters (e.g. water, alcohol content) simultaneously by one single measurement lasting only a few seconds.

Liquid plant extracts are investigated in the transflection mode at thermostated conditions using light-fibre optics, dried parts of plant (flowers, leaves, roots) also in the reflection mode using a sample desk. For the quantitative analysis of secondary metabolites including 3´,4´,5´-trimethoxyflavone in *Flos Primulae veris*, hypericin and hyperforin in *St. John´s Wort*, etheric oils in *Achillea* species, a reference method based on liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE) is applied. Qualitative cluster analysis not only allows identifying different parts of a plant but also enables to distinguish different species, which is essential also in traditional Chinese medicine (TCM).

In the present contribution the main advantages of the novel quality control NIRS tool in medicinal plant analysis are pointed out and discussed in detail by several applications.

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Keywords: Near infrared spectroscopy; Medicinal plant; Transflection; Diffuse Reflection; Multivariate analysis; Cluster analysis

Introduction

Today there is an increasing need for the qualitative and quantitative analysis of medicinal plants since each of them is composed of a great number of beneficial compounds with different pharmacological activity [1,2]. The identification, discrimination and classification of authentic medicinal plants are especially difficult due to its complex sources. Many plants have multiple related species being similar in morphology, cytology and even genome. Moreover, some medicinal products are often mixed or adulterated with other less effective parts of the same plant having no medicinal benefit at all. Many medicinal plants are especially prepared before use according to some guidelines, e.g. those from traditional Chinese medicine (TCM). Different preparation methods including drying, cutting, stir-frying, cooking, etc. may directly affect the quality of medicinal plants and extracts derived there from. In the past, especially traditional medicines were identified and discriminated by experienced personal, which is limited to self experience and ability of determination. During the last years, some computer aided research was carried out to conduct numerous taxonomy researches on the origin of plants which may provide evidence for the classification and identification of the crude medicinal material [1]. Although these methods were found to be helpful, no real break-through was achieved due to the highly complex calculation methods needed to be implemented. Therefore, in the last few years, research was focused on alternative techniques comprising computer supported calculations, mainly multivariate methods, and near infrared spectroscopic techniques [4].

The quantitative determination of a single compound in a plant often leads to a loss of information about the whole plant metabolite because extraction, purification and separation procedures are established to analyze a single compound of interest. So in many cases other pharmacological active ingredients are not accounted playing an important role for the activity [5]. In recent years, the main research was focused on fingerprint techniques, such as gas chromatography (GC) [6], liquid chromatography (LC) [7,8], thin layer chromatography (TLC) [9], capillary electrophoresis (CE) [10,11], capillary electrochromatography (CEC) [12], nuclear magnetic resonance (NMR) [7], etc., which are helpful to give an overall understanding of the pharmacological active ingredients.

Similar to the demand for a novel strategy to fulfill qualitative requirements, the simultaneous determination of multiple compounds needs for alternative analytical techniques. Therefore, we introduced near infrared spectroscopy (NIRS) as a flexible, robust, non-invasive, highly reproducible and high-throughput analytical tool for the qualitative and quantitative characterization of medicinal plants and their constituents.

2. Near Infrared Spectroscopy

In near infrared spectroscopy (NIRS) excitation of molecules is accomplished in a wavelength range between 750 and 2500 nm, corresponding to a wavenumber range between 4000 and 12.800 cm^{-1} [13, 14]. In the infrared region molecules containing C-H, C-O, C=O, N-H and O-H functional groups are excited to stretching-, deformation- and scissor-vibrations, which overtones and combinations can be found in the near infrared region [15]. Spectra are recorded by light interaction with the material of interest and reflection of a part of the reflected light to a PbS-detector, which transforms the optical signal into an electronical. Differences in spectra are very often marginal so that a visual interpretation of overlapping vibration bands is in many cases in praxis impossible. Chemometrics, a mathematical, statistical, multivariate tool is applied for further treatment of recorded spectra (Figure 1).

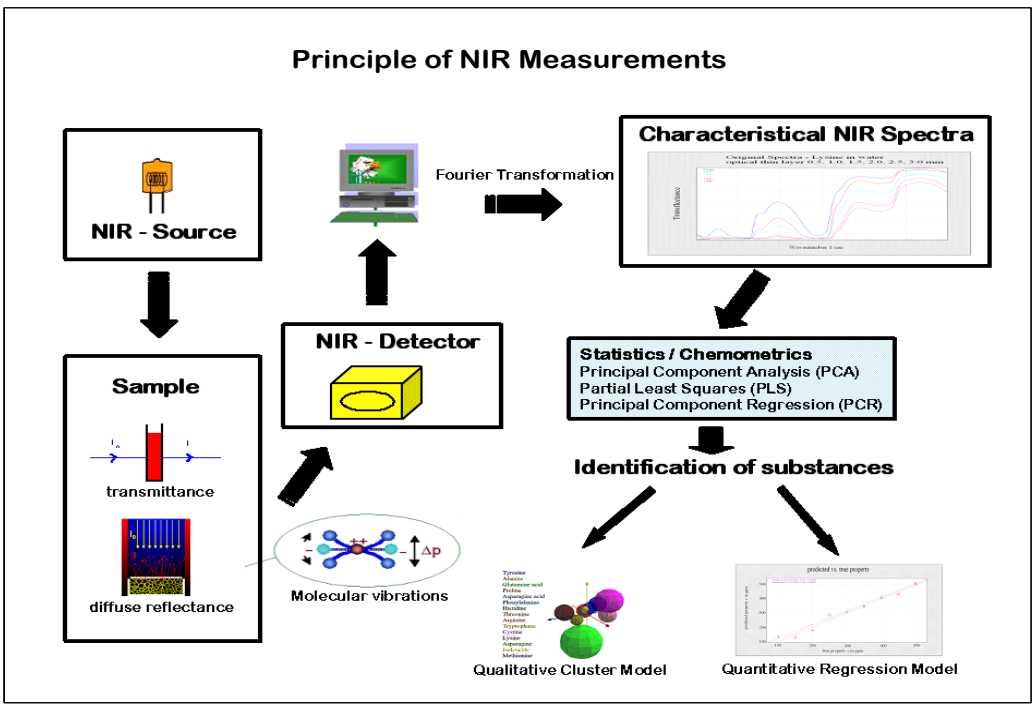

Figure 1. Principle of near infrared spectroscopy (NIRS)

The established, optimised and validated mathematical calibration model can then be used in order to determine the presence and /or the concentration of a certain component [16-18].

The main advantages of the near infrared spectroscopic (NIRS) method for the characterisation of medicinal plants and their components are:

- Short analysis time of only a few seconds enabling high sample throughput
- Non invasive

- No additional materials are required (cheap)
- Simultaneous determination of physical and chemical parameters
- Measurement is possible in the in-line, on-line and off-line mode

Transparent materials such as liquid extracts are usually analyzed by transmission or transflection, solid materials like tissue by diffusive reflectance in suspension mode. In all modes the absorbance relative to a reference is determined.

3. Chemometrics

Applied chemometrical procedures include principal component analysis (PCA) in order to reduce the number of variables to carry out qualitative and quantitative analysis. Data pre-treatment on one hand allows minimizing in-homogeneities originating from the recording of the spectra and enables elimination of shifts in baseline and differences in intensity caused by different sample positioning applying normalization algorithms on the other hand. Diffusion can be compensated by multiplicative scatter correction (MSC). Spectral noise can be reduced by performing the first or second derivative of the original spectrum [19]. Calibration of the NIR spectrometer is carried out in the following steps: 1) Choice of a representative sample set. 2) Recording of the NIR spectra and measuring the reference values. 3) Multivariate modelling, to generate a relationship between the recorded spectra and the reference values. 4) Validation of the system. The most frequently regression methods comprise principal component regression (PCR) and partial least square (PLS).

The choice of the highest suitable regression model is based on the calculation of the following values:

1) BIAS, i.e., the average deviation between the predicted values (y_n) and the actual values (x_n), in the calibration-set, should be close to zero.,

$$Bias = \frac{1}{N}\sum (x_n - y_n)$$

2) PRESS, Predicted Residual Error Sum Square is the sum of the square of the deviation between predicted and reference values. The PRESS value of the validation set should be as small as possible and similar to that of the calibration set.

$$PRESS = \sum (x_n - y_n)^2$$

3) Standard error of estimation (SEE), i.e., the standard deviation of the differences between reference values and NIRS-results in the calibration set.

$$SEE = \sqrt{\frac{1}{N}\sum (x_n - y_n - Bias)^2}$$

4) Standard error of prediction (SEP), i.e., the counterpart for the test-set samples. SEE and SEP should be as small as possible.

$$SEP = \sqrt{\frac{1}{N}\sum (y_n - y_n - Bias)^2}$$

5) The correlation coefficient (R^2) should approach 1.

4. Characterization of Medicinal Plants and Their Constituents

In the following the potential of NIRS for the qualitative and quantitative characterization of medicinal plants and their constituents including secondary plant metabolites and leading compounds down to the ppm-level are discussed. The suitability to analyse simultaneously chemical and physical properties supported by chemometrical approaches is shown by three selected examples: *Flos Primulae veris* is used due to its anti-inflammatory properties, *St. John´s Wort* for the treatment of mild and moderate depressions; *Achillea* is used for the treatment of gastrointestinal diseases.

4.1. Flos Primulae Veris

Flos Primulae veris is used due to its flavonoid content related anti-inflammatory properties and its effect as an expectorans for the treatment of a cold and related sinusitis. In Figure 2 a general strategy scheme for NIR-analysis of compounds in liquid plant extracts is shown. For the control of the *Primulae veris* Flos content the leading compound 3´,4´,5´-trimethoxyflavone was determined by reversed-phase liquid chromarography (RP-LC), which was used as a reference method [20]. After optimization of the temperature and the optical thin layer 220 NIR spectra of 44 charges were recorded in the transflection mode with the spectrometer. Mathematical pre-treatment and statistical analysis were carried out by performing partial least squares regression (PLS). In Figure 2 the possibility to control other plants in a multi plant component extract is illustrated.

Recording the NIR-spectrum (Figure 3a) and calculation of its 1st derivative spectrum allowed to identify characteristic absorption bands. The most intensive band in the spectrum belonged to the vibration of the 2nd overtone of the carbonyl group (5352 cm^{-1}), followed by the C-H stretch and C-H deformation vibration of ethanol (7212 cm^{-1}), the-OH vibration of water and ethanol (4440 cm^{-1}), the -CH_2 overtone (5742 cm^{-1}) and the –CH_2-/-CH_3 overtone (5808 cm^{-1}) (Figure 3b). All recorded spectra were normalized and transformed to their first derivative before calculating the PLS model. Thereby, normalization allowed minimizing shifts in the baseline.

The robustness of the NIRS model is high, which is demonstrated in the similarity of the results for SEE (0.0057) and SEP (0.0099). Accuracy is expressed in the bias. Its value is -3.89% with respect to the mean. So the RP-HPLC results agree with NIRS on average. Studying the influence of the sample temperature showed highest reproducibility at 23°C. Optimisation of the optical thin layer showed highest abundance at 0.5 mm length. Finally a correlation coefficient of 0.95421 for the calibration curve of NIR-values against LC values was established (Figure 4).

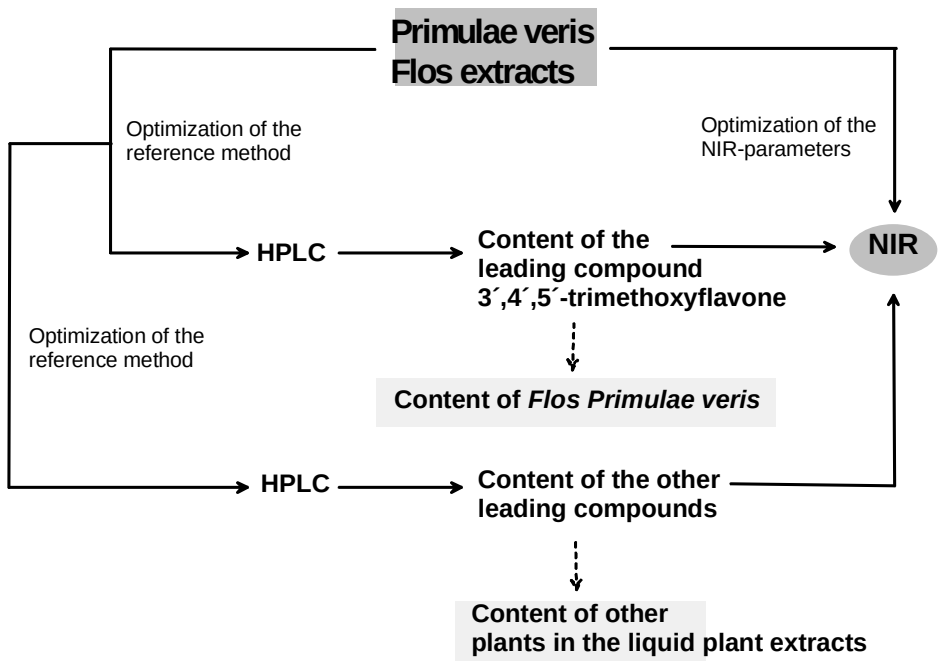

Figure 2. Strategy of analysis of *Primulae veris* Flos extracts

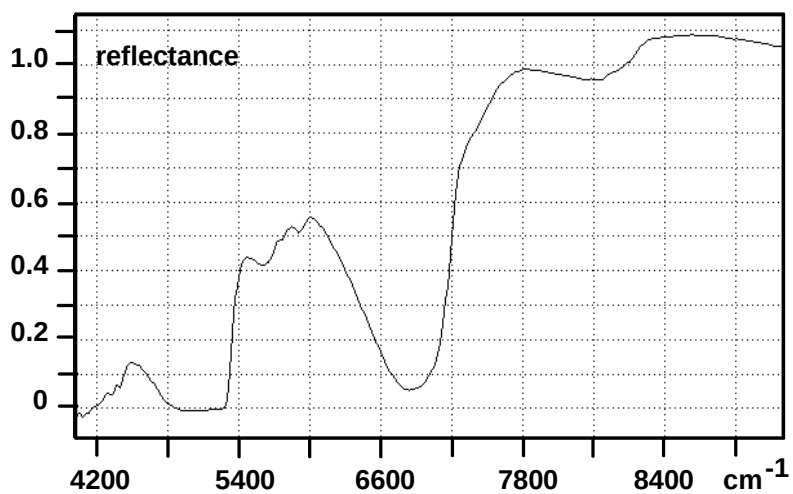

Figure 3a. NIR spectrum of a *Primulae veris* Flos extractive

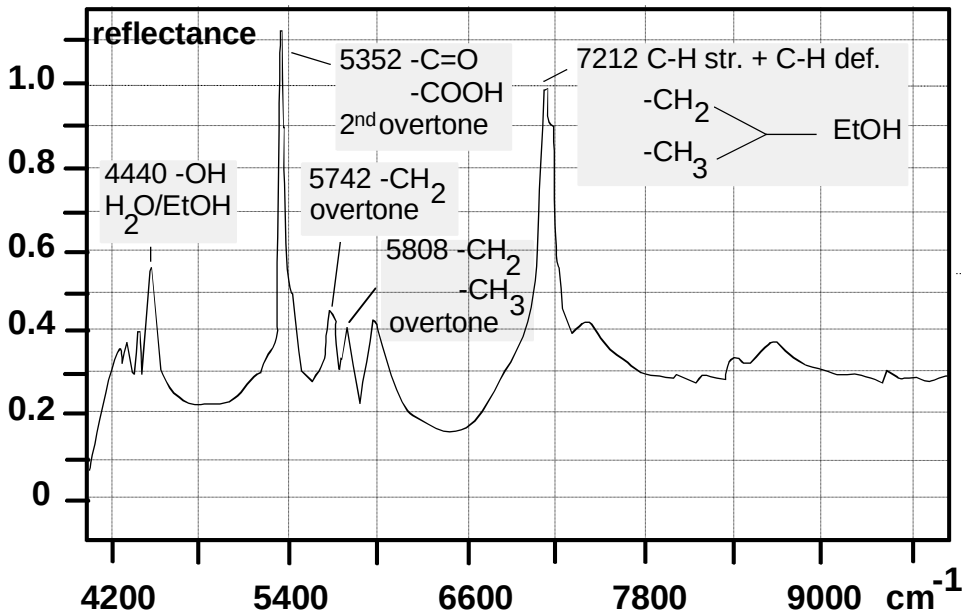

Figure 3b. 1st derivative of the NIR-spectrum with characteristic vibrations

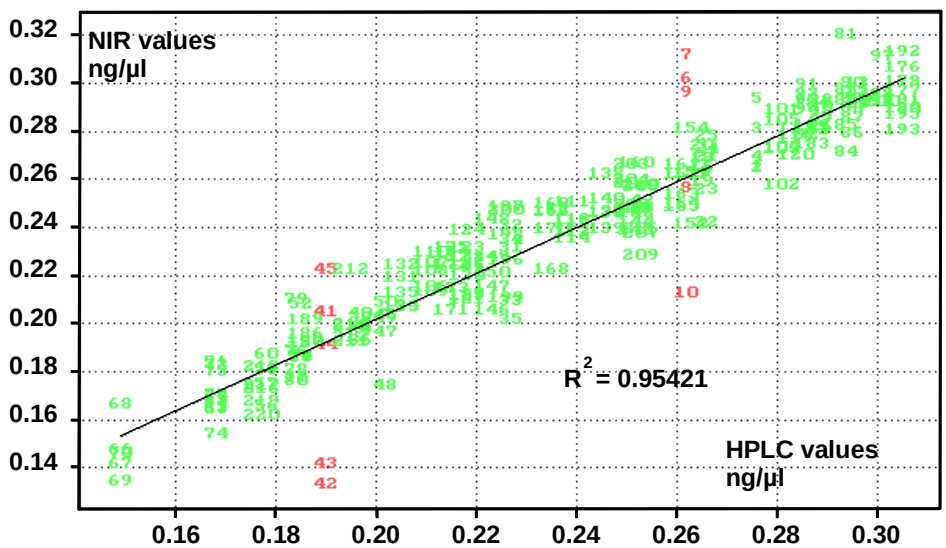

Figure 4. Calibration curve for 3´,4´,5´-trimethoxyflavone. Correlation between LC and NIRS

In order to control the solvent composition a calibration curve with a correlation coefficient of 0.99530 for the determination of the water content was calculated for which the reference data were received by Karl-Fischer titration (Figure 5a). In the same way also the correlation of the ethanol content between the gas chromatographic and the NIRS method showed a coefficient of 0.99701 (Figure 5b).

Validation and results of real samples showed that the robustness and reproducibility of the NIRS model for the determination of the 3´,4´,5´-trimethoxyflavone, water and ethanol content is high (Table 1). The model can be used to predict the content of 3´,4´,5´-trimethoxyflavone in liquid plant extracts with varying matrix composition.

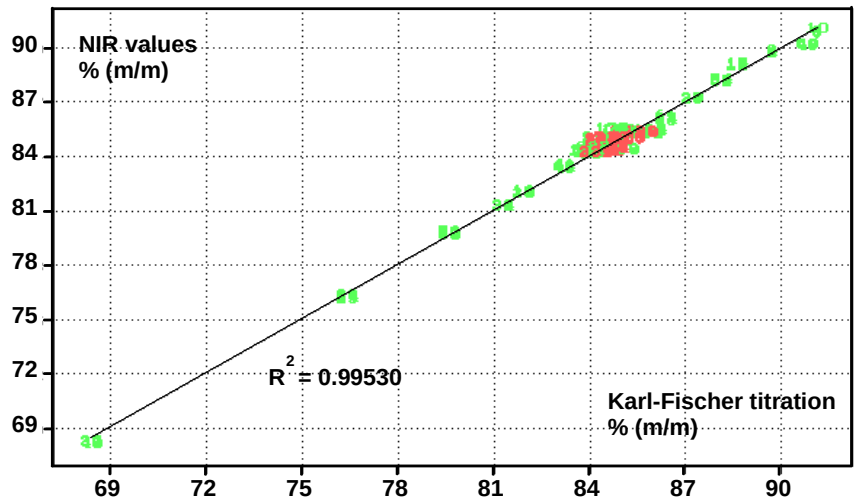

Figure 5a. Calibration curve for water. Correlation between Karl-Fischer titration and NIRS

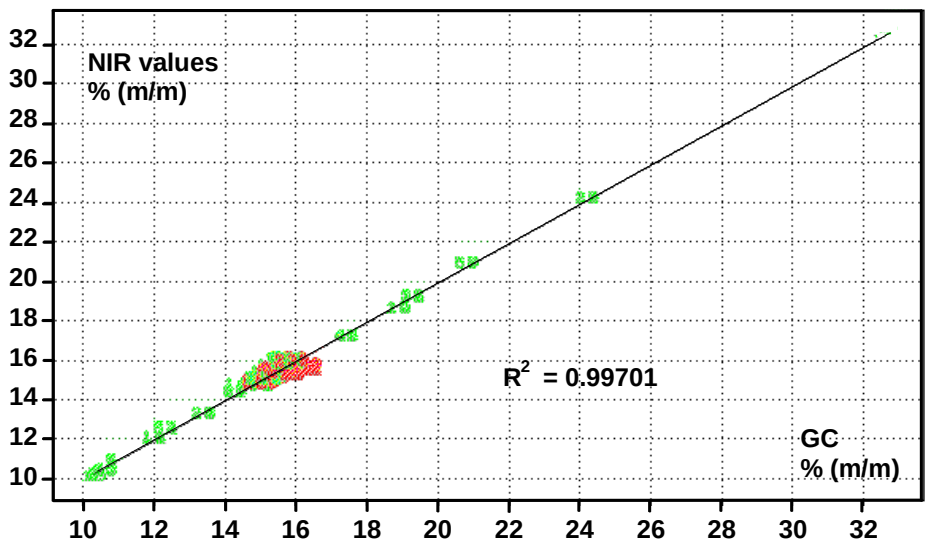

Figure 5b. Calibration curve for ethanol. Correlation between gas chromatography and NIRS.

Table 1. Validation and results of real samples

Ch Charge	HPLC-value: (ng/µl)	NIR-value: (ng/µl)	% H_2O	% EtOH
34	0.219	0.219	80.38	15.7
22	0.222	0.210	80.83	15.2
11	0.197	0.230	80.25	15.7
2	0.210	0.186	81.33	14.5
42	0.245	0.193	79.69	15.7

4.2. St. John´s Wort

St. John´s Wort extract is used for treatment of skin injuries, burns, neuralgia, for its bactiostatic/bacteriocide activity and as a treatment for mild to moderate depression [21-27]. Hypericin and hyperforin are discussed as being the active antidepressant components in *Hypericum perforatum* L. extracts, although it is still unclear how and why St. John´s Wort extract works as an antidepressant [28-33]. It is commonly known that the extract acts as a mild monoamine oxidase inhibitor and a strong serotonine reuptake inhibitor [34]. Hypericin and hyperforin act as standards in the phytopharmaceutical industry. They are the main representatives of the naphtodianthrone group. The lower concentrated cyclopseudohypericine might be synthesized out of pseudohypericin [35]. Beyond the main representatives of the phloroglucine derivative hyperforin, presence of isohypericin, desmethylpseudohypericin, hypericodehydrodianthrone, pseudohypericodehydrodianthrone and skyrin exist [37-39] (Figure 6).

Several analytical procedures have been established including UV-spectroscopy [40], fluorescence microscopy [41], thin-layer chromatography (TLC) [43-45], liquid chromatography (LC) [46-50], LC coupled to mass spectrometry (LC-MS) [51] and capillary electrophoresis [52]. Special sample pre-treatment procedures include liquid-liquid extraction (LLE) and solid-phase extraction (SPE) has been worked out [51]. As all these methods are extremely time-consuming and peak-tailing effects in LC make quantitative determinations difficult, NIRS offers a fast alternative for the simultaneous quantitation of naphthodianthrones and phloroglucines.

Prior to analysis of spectra via NIRS a reference method based on LC, LC-MS and CE must be established to enable quantitation of hyperforin and hypericin [53]. Optimisation of LC parameters finally allowed analysing naphthodiantrones and phloroglucines in one single run (Figure 7).

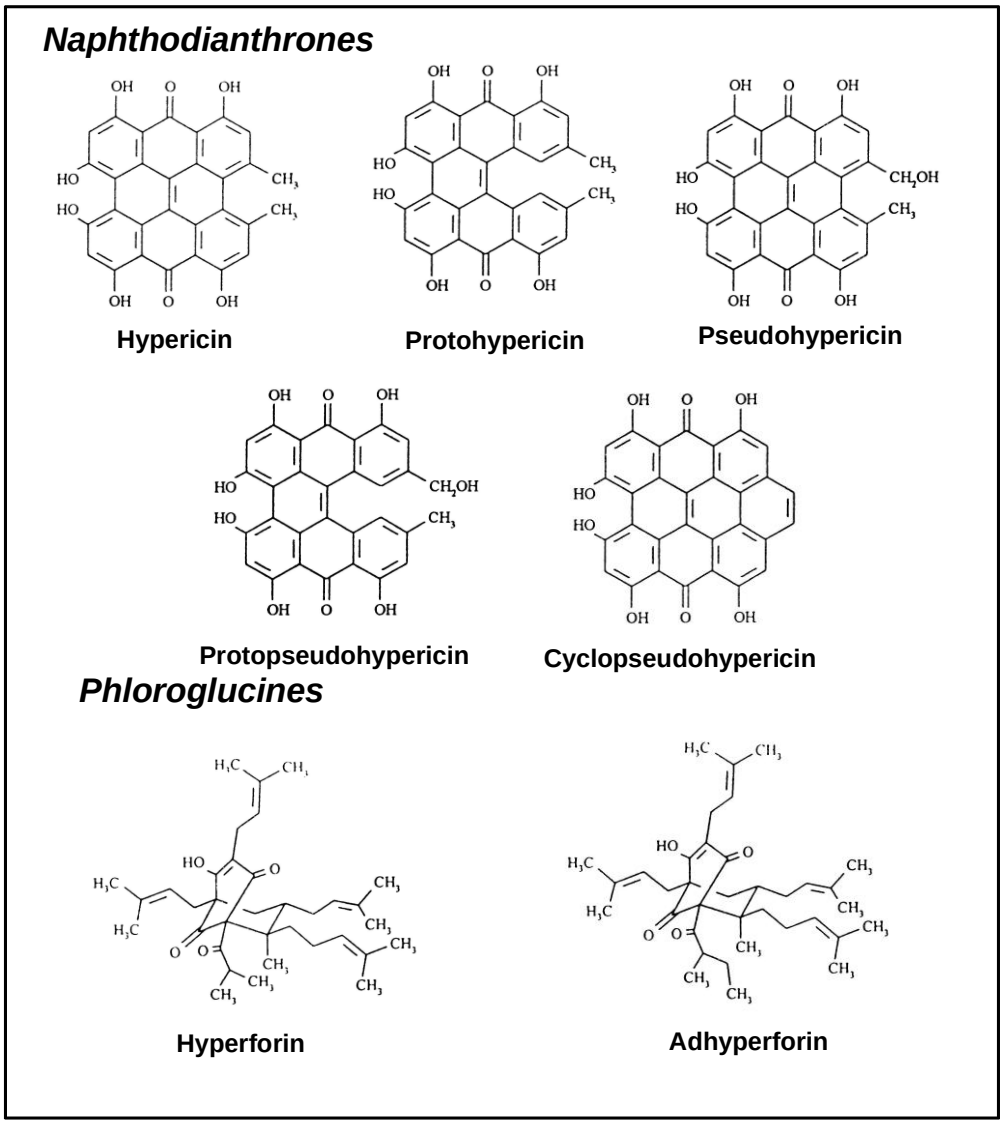

Figure 6. Structural formulae of the most important naphthodianthrones and phloroglucines in St. John´s Wort

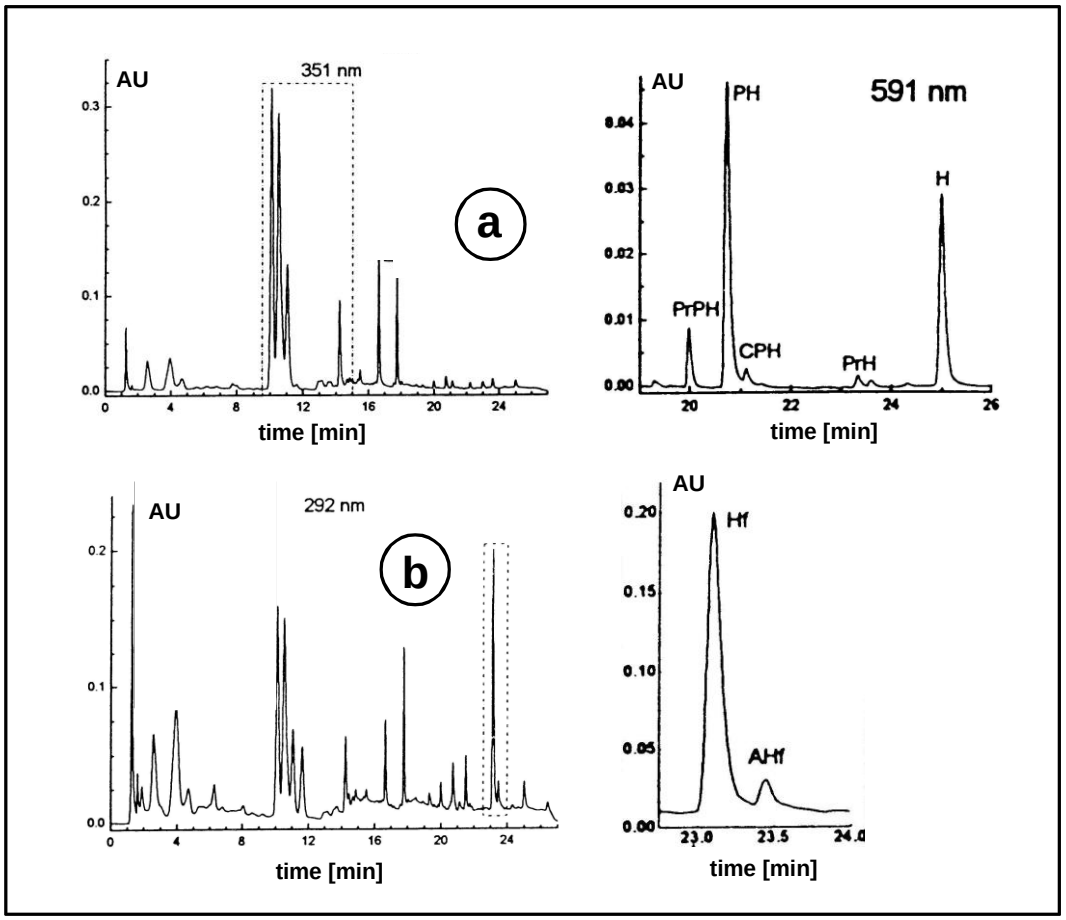

Figure 7. Simultaneous determination of (a) naphthodianthrones and (b) phloroglucines. Conditions: Hypersil BDS-C18 (5 µm, 130 Å, 250 × 4 mm) ; mobile phase, A: 888.0 g buffer (880.0 g bidest, 2 ml 85% H_3PO_4, TEA (pH 2.80)), 80.0 g ACN, B: 49.64 g buffer (50.0 g bidest, 1 ml 85% H_3PO_4, TEA (pH 6.10)), 85.04 g methanol, 275.28 g ACN; gradient, see Table 2; sample volume, 20 µl; peak assignment, PrPH, protopseudohypericin; PH, pseudohypericin; CPH, cyclopseudohypericin; PrH, protohypericin; H, hypericin; Hf, hyperforin; Ahf, adhyperforin.

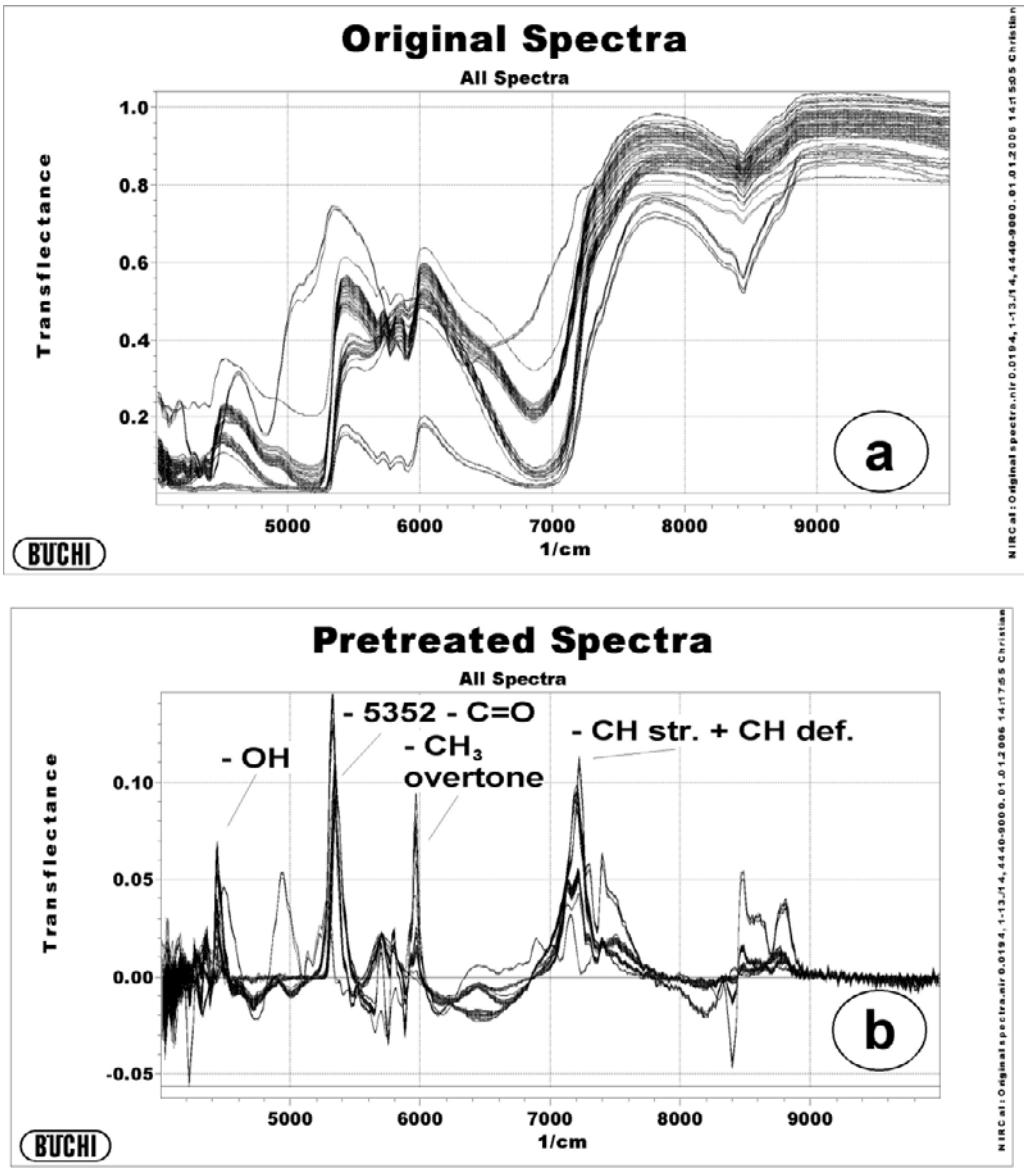

Figure 8. NIRS-spectra of St. John´s Wort extract samples: (a) original spectra, (b) pretreated spectra (normalisation and calculation of its 1st derivative Taylor 3 points); wavenumber range, 4500-9996 cm^{-1}; optical thin layer thickness, 1 mm; scans, 10; temperature, 23 °C.

As an alternative capillary electrophoresis (CE) should enable fast separation and high sensitivity and should allow cross-validation with results obtained from LC studies. Testing several buffer systems, different amounts of modifiers allowed establishing a CE procedure for the separation of hypericin and pseudohypericin within less than 2 minutes [53]. Due to the fact that only the LC method enabled simultaneous analysis of naphthodianthrones and phloroglucines, data generated via LC were chosen as reference values for calibrating the NIR spectrometer. In the following NIR measurements were carried out thermostated at 23°C and an optical pathway of 1 mm. Applying these optimised conditions, 320 spectra of 80

extracts were recorded in transflection mode using light fibre optics over a wavelength range from 4008 to 9996 cm^{-1} with a resolution of 12 cm^{-1}. Ten scans were used for one average spectrum to equilibrate in homogeneities. Figure 8a shows 80 original spectra of St. Johns Wort extract samples. Calculation of the first derivative spectra (Figure 8b) allowed identification of characteristic absorption bands. The most intensive band in the spectrum belonged to the vibration of the second overtone of the carbonyl group (5352 cm^{-1}), followed by C-H stretch and C-H deformation vibration, the –OH vibration (4440 cm^{-1}) and the $–CH_2$ overtone (5742 cm^{-1}). Normalisation allowed for minimisation of the baseline shift. In the following all 80 extracts were analysed four-fold by LC for establishing the quantitative regression model. Seventy percent of the spectra were randomly put into a learning-set and 30% into a validation-set. Five primary factors were necessary to reach the best calibration equation. The robustness of the established NIRS model is high, which is demonstrated in similarity of results for SEE and SEP: 0.52 and 0.50 μg mL^{-1} and 0.64 and 0.71 μg mL^{-1} for hypericin and hyperforin, respectively. Accuracy is expressed in the bias. The values are 1.6 and 4.2E-14. So the LC-UV results correspond to NIRS on average. Calculation of the regression equation for hypericin (Figure 9) and hyperforin (Figure 10) resulted in correlation coefficients of 0.994 and 0.985, which are slightly smaller than the correlation coefficients of the LC-UV method. The model can be used to predict the content of hypericine and hyperforine in liquid St. John´s Wort extracts. Results show the possibility for phytopharmaceutical industry to replace LC method, usually applied to determine hypericin and hyperforine in the routine analysis, with NIR method, guaranteeing a high degree of robustness and reproducibility. Due to less sensitivity of the NIRS method, it is evident that for the analysis of the lower concentrated naphthodianthrones proto-, pseudo-, protopseudo-, and cyclopseudohypericine as well as for the phloroglucine adhyperforine in particular LC must be preferred. CE was found to be less reproducible. NIRS has the great advantage of ensuring high sampel throughput and reduced costs. NIRS is a full spectrum method. It cannot only determine the content of the naphthodianthrones and phloroglucines but also of other analytes within one single measurement. Finally, also information about other parameters concerning the quality of the extract, e.g. the solvent composition, etc., can be gained.

4.3. Achillea Genus

The genus *Achillea* exhibits extraordinary ecological amplitude and ranges with great morphological variation. It is hard to identify and discriminate its species due to its highly morphological diversity and chemical homogeneity. Some species, such as *Achillea millefolium, ceretanica, collina, pratensis,* etc., were recognized as one group, the *Achillea millefolium* complex, for their high homogeneity and successful crossing [54,55]. According to the species index of NCBI taxonomy, there are 65 species of *Achillea*, e.g. *clypeolata, collina, millefolium, nobilis, wilsoniana*, etc. A large number of *Achillea* species is endemic and restricted to certain regions in Europe or certain temperature areas in Asia. Only few of the other species are growing over a wide geographical range.

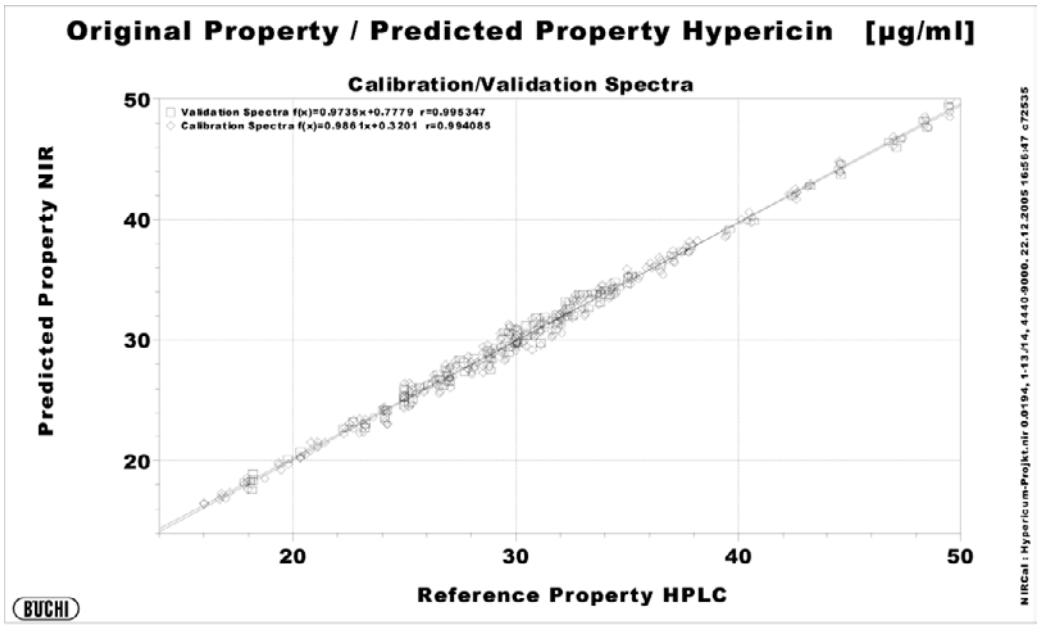

Figure 9. Predicted (NIRS) vs. true property (LC) for the determination of µg/ml hypericin in liquid St. John´s Wort extracts (n=80). R^2=0.99; SEP=0.68

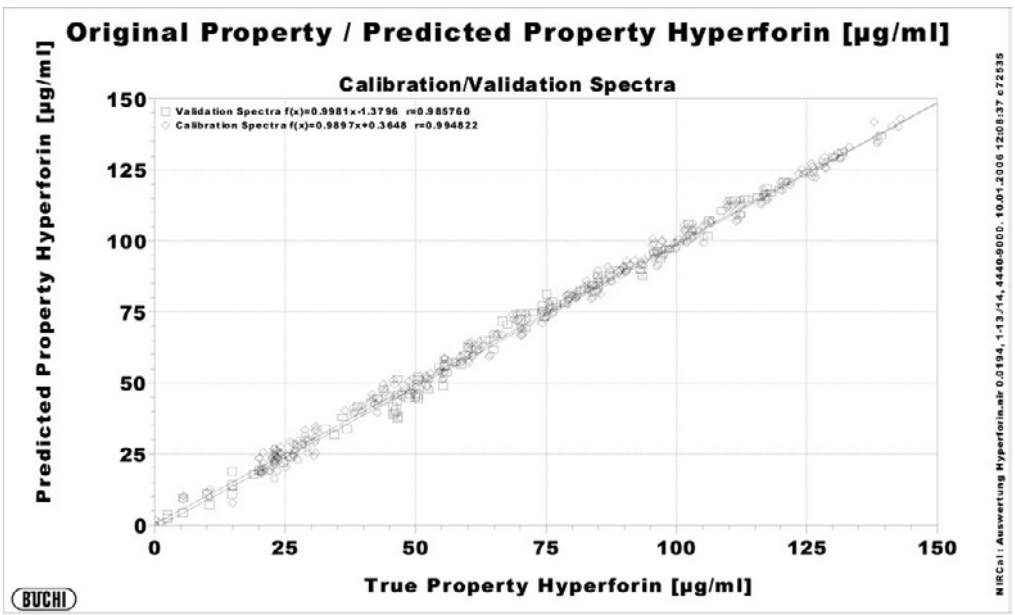

Figure 10. Predicted (NIRS) vs. true property (LC) for the determination of µg/ml hyperforin in liquid St. John´s Wort extracts (n=80). R^2=0.99; SEP=0.72

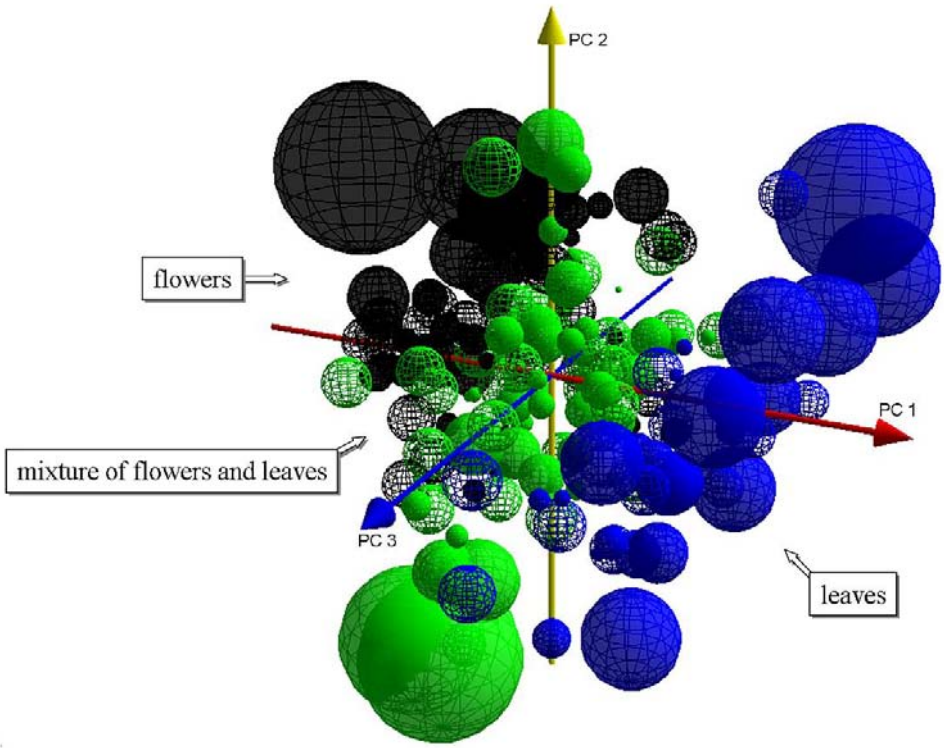

Figure 11. 3-dimensional factor plot, representing principal components (PC) one, two and three, for classifying the aerial parts of *A. millefolium s.l.*.

In our study, we investigated *Achillea millefolium* L and three of its related species. The main objective of this GC-MS supported NIRS study was (1) to discriminate between *Achillea millefolium* flowers and leaves, (2) to differentiate between varying sample preparation procedures (air-dried, oven-dried) and (3) to classify in the future *Achillea* species by means of NIRS combined with multivariate data analysis (MVA). Recording 240 spectra (75 spectra for air-dried flowers, 75 for air-dried flowers and leaves) allowed discriminating by cluster analysis (Figure 11).

The averaged and normalized (between 0 and 1) absorbance spectra are displayed in Figure 12.

In a quantitative NIR study partial least square regression (PLSR) was used to create 14 single-compound models (SCM, one regression model for each compound) on one hand and one multi-compound model (MCM, one regression model for 14 compounds) on the other hand. The averaged standard error of prediction (SEP) showed 0.49 % for SCM and 0.62 % for MCM. Differences between SCM and the MCM for the quantities of the compounds in *Achillea millefolium* were compared by a t-test and one way analysis of variance (ANOVA). Furthermore, the MCM was used to optimize and evaluate the best suitable sample extraction procedure for the raw plant material prior to NIR analysis, namely air-dried, oven-dried and a mixture of both extracts. Gas chromatography - mass spectrometry (GC-MS) was used as reference technique to quantitatively calibrate the NIR system. Multivariate data analysis (MVA) in form of Pearson bivariate correlation, principle component analysis (PCA) and

hierarchical cluster analysis were conducted to uncover the interrelations of the analyzed chemical compounds present in the samples. According to the Pearson bivariate correlation analysis most of the 14 compounds were significantly correlated (Figure 13).

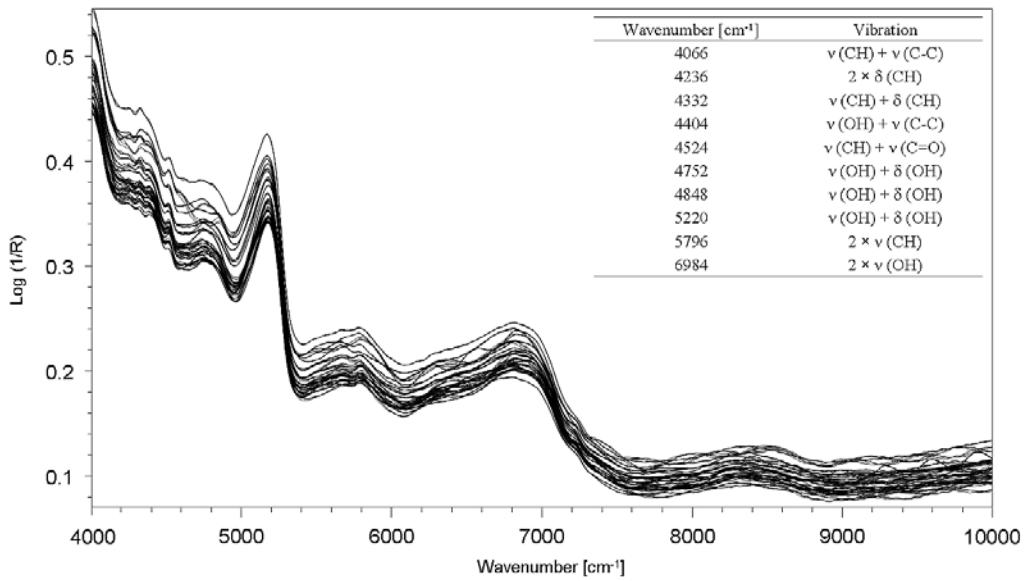

Figure 12. NIR absorbance spectra of the grinded *A. millefolium* plants marked with characteristic vibrations; ν = stretching vibr.; δ = bending vibr.; 2 × = first overtone.

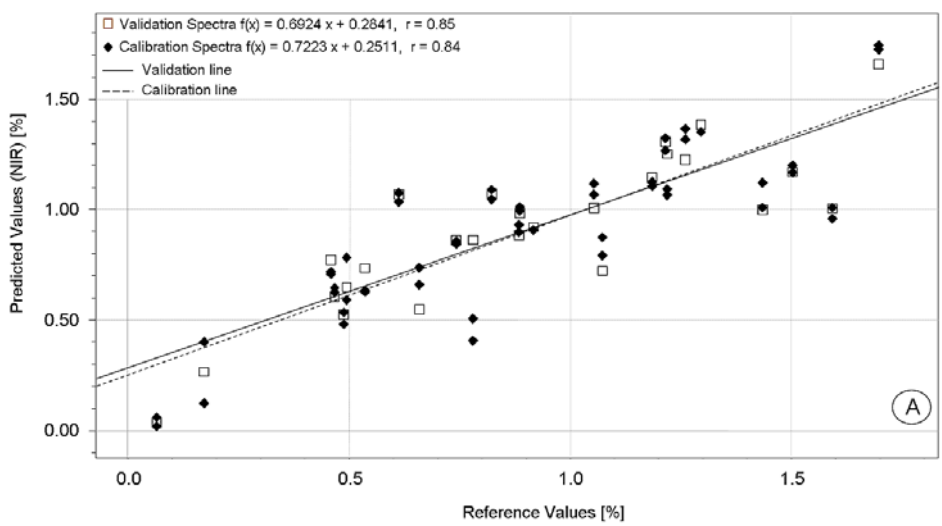

Figure 13. PLS regression lines of the MCM model for determining the n-decanoic acid content in the *A. millefolium*.

Conclusions

It was shown that NIRS offers a huge potential for the qualitative and quantitative analytical characterization of different medicinal plants and their constituents deriving from manifold sources. Chemical parameters can be analysed simultaneously with physical. Due to the short analyses times the method is highly suitable to high-sample throughput and therefore of interest for the phytopharma industry.

Abbreviation List

CE	Capillary electrophoresis
GC	Gas chromatography
LC	Liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PLSR	Partial least square regression
RP	Reversed phase
TCM	Traditional Chinese medicine
TLC	Thin layer chromatography

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Chapter 11

What is the Future of Phytotherapy? (Commentary)

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Practically all human societies have utilized plants not only as sources of nutrition but also as therapy against diseases and ailments. Considering the fact that the synthesis of a pharmaceutical requires an enormous investment of research and money, the discovery of useful medicinal plants which have been used for millennia is very appealing. About 25% of all synthesized drugs are derived directly or indirectly from plants [1].

In the USA the market in plants used for medicinal purposes involved $4.8 billion in 2007 and $5 billion in Europe in 2003 (2-3). The increase in the demand for phytotherapeutic products in the USA has resulted in new rules starting in 2008 so that products adhere to Good Manufacturing Practices (2).

The European Union in 2004 passed a law permitting a simplified registration procedure for herbal medicines which have been used for at least 30 years (and 15 years in Europe). These phytotherapeutic products must have adequate documentation of nontoxicity with specific conditions of use (3).

In the 1990s the World Health Organization (WHO) stated that the use of traditional medicines was the only sustainable way to provide primary healthcare to individuals in developing nations [4]. An international meeting of 134 nations at Alma Ata in 1978 established the objective of providing adequate healthcare for all people in the world by the year 2000. In that year, non-governmental organizations from 92 nations met in Savar, Bangladesh to reaffirm the same goal.

Given this global situation, the patenting of plant medicines by pharmaceutical industries can constitute a problem. A pharmaceutical industry would be reluctant to engage in the high economic investment needed to carry out chemical, pharmacological and clinical studies without an economic endpoint or profit. The only reason that a pharmaceutical industry

would be interested in investing in research in the area of herbal medicines would be a public refundability.

Some of the problems involved with the use of herbal medicines can be summarized as follows:

1. Knowledge of the substances comprising the plant and their actions is incomplete and thus there are subsequent problems of standardization. For example, for years the therapeutic activity of St. John's wort was thought to be due to its content of hypericin but now it appears that its hyperforin content is actually more relevant [5].
2. The composition of an herbal medicine differs according to which part of the plant is utilized, the type of soil in which it grows and the time of the year when it is harvested. For example, the concentration of valerianic acid in *Valeriana officinalis* can vary 100-fold depending on the zone from which it is collected [6].
3. The pharmacological activity of a plant is different from that of its single components. For example, the antioxidant activity of ascorbic acid contained in *Rosa canina* is higher than in ascorbic acid itself due to the presence of carotinoids and flavonoids which potentiate its activity [7].
4. Combinations of plants administered together can modify the bioavailability and therapeutic activity of single active ingredients.
5. Plants can be contaminated by toxic substances (for example heavy metals, aflatoxin, etc.) as well as pathogenic microorganisms.
6. The pharmacological activity of a phytotherapeutic product depends upon the extractive technique used. For example, to treat chronic venous insufficiency, only the triterpenic component of *Centellae asiaticae* is used, as it is comprised principally of asiaticoside (dry extracts) rather than ethanolic extracts, or as powdered Centella herb which has activity on the central nervous system [8].
7. Little attention in the medical history about phytotherapic products due to the phenomenon of self-prescribing.

   Due to the frequent use of herbal medicines, physicians should always ask their patients if they are taking any phytomedicines to avoid cross reactions with other types of drugs [9]. There are have been two reported cases of acute rejection of heart transplant due to combined use of cyclosporin and hypericin self prescribed in one case and prescribed by a psychiatrist in a second case [10]. Hypericin increases the activity of the isoenzyme CYP3A4 of cytochrome P450 and inhibits the absorption of substances and drugs through its action on the P-glycoprotein drug transporter, thus causing decreased bioavailability of cyclosporin and consequent reduced immunosuppressive action.

   Another interaction which can be dangerous is that of co-administration of an anticoagulant and certain herbs such as gingko, papaya, etc. due to risk of hemorrhage. Furthermore, herbal medicines usually do not interfere in the coagulative cascade but on platelet function so with prothrombin and partial thromboplastin times being unmodified, there is a prolonged period of hemorrhage [11].

8. The common idea that "herbs do not harm us because they are not toxic" results in an underestimation of the problems they can create. They are not always "natural and safe" even though many people believe that they are [12]. It is interesting to note the results of a survey conducted in Germany in 2002 on 2172 people ranging in age from 16-90 years: 82% of those interviewed maintained that "natural remedies" are not very toxic [13], whereas 85% of participants in the study believed that chemical drugs were endowed with medium to high toxicity.
9. All of the above makes the carrying out of randomized, controlled studies difficult. Therefore a variation of the CONSORT checklist for the correct use of randomized studies has been proposed [14].

As can be seen by the above mentioned, there are many problems tied to the standardized and scientifically correct use of plant medicines. On the other hand, how can we humans not use this "treasure" which the natural world has made available to us? What is needed is for various national governments and the World Health Organization to sponsor studies on the composition, therapeutic properties and proper use of plant medicines. Up until 2007 there are have been published three volumes of the WHO *Monographs on Selected Medicinal Plants,* containing 91 monographs. The fourth volume with another 28 monographs is in press now. Even given the breadth of these volumes, there are still thousands of plants which have not been adequately studied!

In conclusion, given the difficulties in the correct use and knowledge of plants, it is a pity that this field sometimes is left to therapeutic improvisation and in the hands of incompetent individuals who are not qualified to determine therapies. Both physicians and pharmacists should be adequately prepared during their academic studies in the field of phytotherapy so that they can properly guide therapy for their patients.

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Chapter 12

Quality Control of Polysaccharides from Medicinal Plants and Fungi*

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Abstract

Polysaccharides isolated from medicinal plants and fungi exhibit multiple pharmacological activities, including anti-tumor, anti-oxidation, hypoglycemic activity and immune potentiation and so on. The biological activities of polysaccharides depend on their chemical characteristics. However, quality control of polysaccahrides is a challenge because of their complicate structure, macro-molecular mass, more characters showed relationship with the bioactivities and more potential symbols could be used as the evaluation indicators. In this review, qualitative assay including the tests of purity, molecular weight and its distribution, constituent monosaccharide composition and the ratio, the features of glycosidic linkages, as well as quantitative analysis of polysaccharides from medicinal plants and fungi were reviewed and discussed. Among the various means for quality control of polysaccharides, chromatographic and electromigratic methods including high performance liquid chromatography (HPLC) such as high performance anion exchange chromatography (HPAEC), size exclusion chromatography (SEC) and electrophoresis (e.g. capillary electrophoresis and gel electrophoresis) are powerful techniques. The perspective for quality control of polysaccharides has also been described.

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Keywords: Polysaccharides; Quality control; Medicinal plants; Medicinal fungi; Qualification; Quantitation

1. Introduction

Medicinal plants originate from almost every part of the globe. Such plants serve the primary healthcare needs of up to 80 percent of people in Africa, and account for 30%-50% of the total medicinal consumption in China [1]. WHO has identified 20,000 species of medicinal plants for screening [2]. On the other hand, there are at least 270 species of mushroom (fungus) that are known to have various therapeutic properties [3]. Furthermore, it is reported that there are about 650 species from 182 genera of higher Hetero- and Homo-basidiomycetes (fungi) have been shown to contain biologically active antitumour and immunostimulative polysaccharides [4].

Polysaccharides, a class of carbohydrates consisting of a number of (usually more than 10) monosaccharides joined by glycosidic bonds in branched or unbranched chains, are usually considered as one of the active compounds in medicinal plants and fungi. In last decade, the polysaccharides have attracted a great deal of attention in the biomedical arena because of their broad spectrum of therapeutic properties and relatively low toxicity (Figure 1). It was reported that there are more than 300 kinds of polysaccharides extracted from the natural plants [5], in which the water-soluble polysaccharides from traditional Chinese herbs are most important for their significant pharmacological activities, such as anti-cancer, anti-inflammation, immune potentiation and blood sugar reduction (Table 1). However, the activities of polysaccharides are strongly related to their monosaccharides composition, molecular mass, configuration and position of glycosidic linkages, etc. Therefore, quality control of polysaccharides is necessary for ensuring their efficacy and safety. In this review, qualitative and quantitative analysis, including the tests of purity, molecular mass and its distribution, monosaccharides composition and their ratio, configuration and position of glycosidic linkages, and quantitative determination of polysaccharides were reviewed and discussed.

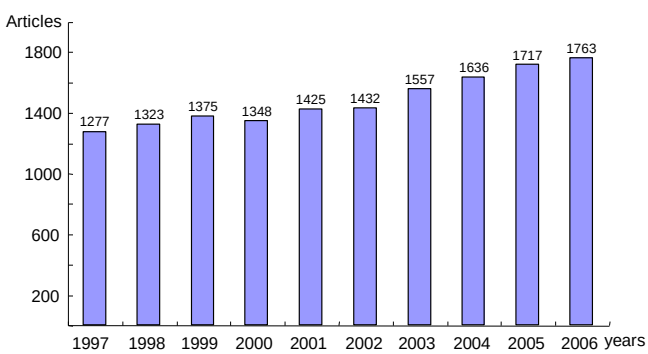

Figure 1. Growth in the number of journal articles on polysaccharides appearing annually during the last decade based on the data from *ISI Web of Science*.

Table 1. The investigated pharmacological activities of polysaccharides from medicinal plants and fungi (Data from 146 journal articles collected in Pubmed dated 2002-2007)

Origins	Immuno-modulation	Anti-tumor	Anti-oxidation	Hypoglycemic activity	Anti-inflammatory	Others
Medicinal Plants						
Acanthopanax koreanum	+					
Acanthopanax senticosus	+					
Achyranthes bidentata						Anti-aging
Aconitum carmichaeli Debx.	+					
Aloe vera L. var. chinensis (Haw.) Berg	+		+			
Aloe barbedensis Miller			+		+	
Angelica sinensis	+	+	+			Cytoprotection, anticoagulation
Angelica gigas Nakai	+	+				
Artemisia capillaris						Anti-adhesive effect
Astragalus membranaceus	+		+	+	+	Alleviation on keney injury, antibiosis
Brassica napus L.		+				
Bergenia crassifolia (L.) Fritsch	+					
Bupleurum kaoi						Hepatoprotection
Bupleurum smithii	+					
Cactus		+				
Camellia sinensis						Antibiosis
Cistanche deserticola Y.C. Ma	+					
Conyza canadensis			+			Anticoagulation
Echinacea angustifolia	+					
Eleutherococcus senticosus						Hepatoprotection
Euphorbia kansui			+			
Ginkgo biloba		+				
Glinus oppositifolius (L.) Aug. DC.	+					
Glycyrriza uralensis	+					
Isatis	+					
Juniperus scopolorum	+					
Lemna minor L.					+	
Litchi chinensis Sonn.			+			
Lpomoea batatas	+					
Lycium barbarum	+	+	+	+		Radioprotection, anti-myelosuppression, anti-aging, cytoprotection
Maytenus ilicifolia					+	
Menyanthes trifoliata L	+					
Ocimum sanctum			+			Radioprotection

Table 1 (Continued)

Origins	Immuno-modulation	Anti-tumor	Anti-oxidation	Hypogly-cemic activity	Anti-inflam-matory	Others
Panax ginseng	+		+		+	Radioprotection, antibiosis, anti-adhesive effect, hepatoprotection, anti-septicemia
Panax quinquefolius L.	+			+		
Phleum pretense L.	+					
Phyllanthus niruri	+					
Prunella vulgaris L.	+					
Pumpkin				+		
Punica granatum			+			
Rheum tanguticum			+		+	
Rosa rugosa			+			
Salicornia herbacea	+					
Sophora subprosrate			+			
Medicinal Fungi						
Agaricus blazei			+			
Antrodia camphorata					+	
Antrodia cinnamomea						Hepatoprotection
Cordyceps sinensis	+	+	+	+		
Cordyceps ophioglossoides		+				
Cordyceps mycelia			+	+	+	
Collybia dryophila	+				+	
Coriolus versicolor	+	+			+	Cancer chemoprevention
Ganoderma lucidum	+	+	+		+	Anti-angiogenesis, antiherpes, neuroprotection, healing efficacy, antiulcer, hepatoprotection, cancer chemoprevention
Lentinula edodes	+	+				Antibiosis, cancer chemoprevention
Lyophyllum decastes Sing.		+				
Pleurotus abalonus			+			
Pleurotus citrinopileatus		+		+		
Pleurotus ostreatus	+	+				
Polyporus albicans (Imaz.) Teng	+					
Poria cocos	+					
Schizophyllum commune	+	+				
Tremella fuciformis	+					Antibiosis

2. Isolation and Fraction of Polysaccharides

Plant and fungal cell walls are primarily polysaccharide in composition. Selection of an extraction method, the first and usually the most important process for quality control, depends on the cell wall structure. Hot water extraction has been a popular approach. In general, the extraction method involves elimination of low molecular substances from sample material with a certain organic solvent, followed by the extraction with water (100 ^{o}C) for certain time. This extraction yielded water-soluble polysaccharides. The parameters such as extraction time, solid/liquid ratio, immersing time and extraction temperature were studied to optimize the polysaccharides extraction using uniform design [6]. Actually, the extraction method can be varied based on the structure and water-solubility of polysaccharides, but the basic rule is to break the cell wall from outer layer to the inner layer with mild-to-strong extraction conditions (pH and temperature). Therefore, alkali solutions are usually used for extraction of acidic polysaccharides which are insoluble in hot water [7-10]. It has also been noted that hot-water extraction of polysaccharides is associated with long extraction time and high temperature. Therefore, it is desirable to find a method for economical and efficient extraction of polysaccharides. The advantages of ultrasonication treatments during the extraction of polysaccharides have been documented for various types of plant tissues [11-14]. However, the high extraction efficiency of ultrasonication is spotted because it may degrade some polysaccharides [15].

Pulsed electric field electro-technology (PEF) is an emerging technology in the field of food preservation, which is based on a pulsing power delivered to the product placed between a set of electrodes that confine the treatment gap of the PEF chamber [16]. Under the process of PEF, the differential electric pressure between the cell interior and the exterior of cell membranes is so large that it will lead to rapid permeation. Consequently, the concentration between the cell interior and the exterior of cell membranes can reach equilibrium in an ultrashort time. PEF has been used for the extraction of active ingredients such as polysaccharides from natural biomaterial at room temperature without any heating process [17]. The result showed that the largest extraction ratio is 55.59% by PEF on the conditions of 0.5% KOH, 20 kV/cm electric field intensity and 6 μs pulse duration. Comparing it with the conventional extraction methods, such as alkali extraction method, enzyme extraction method and compound extraction method, the extraction ratio and polysaccharide content of PEF method are higher than the other three methods, while the impurity of extraction material is less. So the PEF method is a novel and promising method to extract polysaccharide, which will be beneficial to the food and drug industries.

Extracted polysaccharides can be further fractionized and purified using a combination of techniques, such as precipitation (ethanol, fractional and acidic precipitation), ultrafiltration, gel filtration, ion-exchange chromatography and affinity chromatography. The separation of water soluble and insoluble polysaccharide can be achieved by successive extraction of raw material with hot water and different alkaline solutions. The impurities of hot water extract are excluded using ethanol precipitation. Then the separation of acidic and neutral polysaccharides can be achieved by anion-exchange chromatography. The neutral polysaccharide in the mixture is first eluted by an appropriate running buffer; the acidic polysaccharide is then eluted at a higher salt concentration. Different molecular sizes of

polysaccharides are separated by gel filtration (Figure 2). Ultrafiltration is also used for fractionation of polysaccharides with different molecular size or for removal of low molecular weight compounds coextracted.

3. Qualitative Analysis of Polysaccharides

Qualitative analysis is used to distinguish the polysaccharides using their structural, physical and chemical properties.

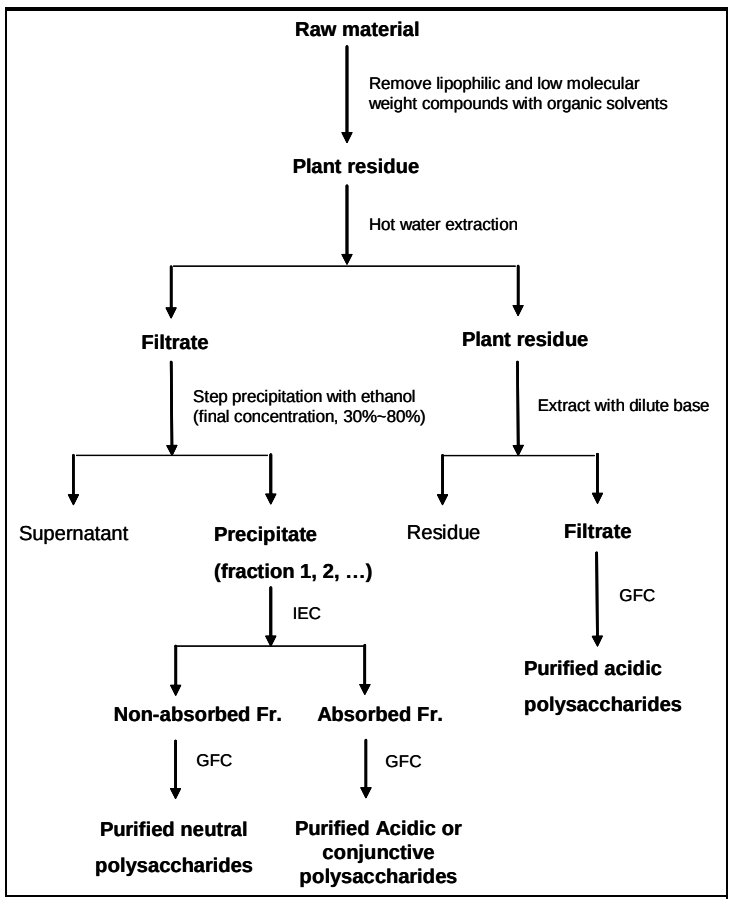

Figure 2. Diagrammatic scheme of isolation, fractionation and purification of polysaccharides from raw materials IEC, ion-exchange chromatography; GFC, gel filtration chromatography.

3.1. Purity

Purity is crucial for determination of polysaccharide's properties. Because its complex and specification, the purity elucidation of polysaccharides is very different from the low molecular weight compounds. For example, melting point and solubility are usually not

available, while viscosity [18,19] and refractive index (RI) [20-22] can be used for determining the purity of polysaccharides. Though the homogeneity of purified polysaccharides can be indicated by the stable optical rotation achieved in aqueous solution at a certain temperature [23-24], the variations are great depending on the conditions so the method isn't decisive. Therefore, chromatographic methods including paper chromatography (PC) [25], thin layer chromatography (TLC) [26], gel permeation/filtration chromatography (GPC/GFC) [27,28], size-exclusion chromatography (SEC) [29], as well as electrophoresis [19,27] are widely applied for more decisive qualitative analysis [30-36] (Table 2). Diffusion ordered spectroscopy (DOSY) is also a rapid method for verifying purity of polysaccharides [37]. Nuclear magnetic resonance (NMR) spectroscopy can provide the structural characteristics of polysaccharides, which is sensitive for the purity test [38,67]. In general, at least two methods should be used for purity assay in order to obtain an appropriate result.

Table 2. The methods for purity test and molecular weight (MW) measurement of polysaccharides

Techniques	Methods			Propose	Ref.
	Solvent	Column	Detection		
HPLC					
	H_2O	Ultrahydrogel 500 or Shodex SUGAR KS-805	UV	Purity	30, 43, 50
	DMSO, H_2O, HAc- NaAc buffer, $LiNO_3$, NaCl or $NaNO_3$	Plaquagel–OH, Sepharose 6B, Shodex OHpak, TSKgel series, Ultrahydrogel series or Waters Hsp-Gel AQ5.0	RI, UV, LLS, RALLS, LALLS, MALLS or Visco-meter	MW	18, 23, 27, 91, 100, 103, 104, 118-131
	H_2O, Na_2SO_4, NaCl, Na_2Ac, NaOAc, $NaNO_3$, $NaNO_3$ and NaH_2PO_4 or Phosphate buffer	Biosep SEC, Shodex OHpak and SUGAR, TSKgel series, Ultrahydrogel series or Superose 6 HR	RI, UV, ELSD or MALLS	Purity, MW	20-22, 24, 34-36, 40-42, 44-66
GCC					
	NaCl or H_2O	Sephadex gel	Phenol–H_2SO_4 assay	Purity	26, 27, 40
	H_2O or DMSO	Sepharose, Sephacryl, Toyopearl, Sephadex, Superdex, Plaquagel- OH or TSKgel series	RI , Phenol–sulfuric acid method or MALDI-TOF MS	MW	25, 28, 43, 83, 132-140
PC					
	Not mentiond	Whatman Nos. 1 and 3 mm sheets	Alkaline silver nitrate regent	Purity	25
TLC					
	Not mentiond	Cellulose plate	Silver nitrate solution	Purity	26
High-voltage PE					

Table 2. (Continued)

Techniques	Methods			Propose	Ref.
	Solvent	Column	Detection		
	Not mentiond	Whatman No. 1 filter paper	Periodic acid and fuchsin-sulphurous acid regent	Purity	27
CE					
	Boric acid–KOH buffer	Uncoated fused-silica capillary	UV 254 nm	Purity	42
LLS					
	H_2O, NaCl and LiCl/Me_2SO	-	-	MW	30,141
NMR					
	D_2O	-	DOSY	Purity, MW	37, 67

HPLC, High performance liquid chromatography; **GCC**, Gel column chromatography; **TLC**, Thin layer chromatography; **PC**, Paper chromatography; **High-voltage PE**, High-voltage paper electrophoresis; **CE**, Capillary electrophoresis; **LLS**, Laser light scattering detector; **NMR**, Nuclear magnetic resonance; **SEC**, Size-exclusion column; **RI**, Refractive index; **UV**, Ultraviolet; **ELSD**, Evaporative light scattering detector; **MALLS**, Multiangle laser light scattering detector; **RALLS**, Right angle laser light scattering; **MALDI-TOF MS**, Matrix-assisted laser desorption/ionization-time of flight mass spectrometery; **DOSY**, Diffusion ordered spectroscopy.

3.2. Molecular Weight and the Distribution

The molecular size of polysaccharides is an important physico-chemical parameter which correlates with its biological activity. It was reported that levan antitumour activities depend on the polysaccharide molecular weight and that a specific class of molecular weight may be responsible for this effect [39]. The moderate molecular mass of the polysaccharides from *Poria cocos* sclerotium also contributed beneficial to enhancement of antitumor activity [68]. The similar results were also obtained in other studies [69-73]. Therefore, the determination of the molecular mass is very important for quality control of polysaccharides.

There are several concepts for molecular mass of polysaccharides, which is different from that of small molecule compound. They are the peak average molecular weight (Mp, average molecular weight at peak apex), the weight average molecular weight (Mw) and the number average molecular weight (M*n*). The ratio of Mw to M*n* (Mw/M*n*) is an indicator of molecular weight homogeneity for the sample, which is a measure of the width of the molecular weight distributions, and the higher the ratio, the greater the width of the distribution. In general, a homogenous material will have a ratio of 1.0. Several techniques have been applied for the determination of molecular mass of polysaccharides. They are mass spectrophotometry, osmotic pressure method, vapor pressure method, analytical ultracentrifugation, viscosity measurements [74], light scattering [75], high performance capillary electrophoresis (HPCE) [76], HPLC [77-79] and NMR [36], etc. One useful technique that is capable of investigating whole molar mass distribution is SEC, which is also known as GPC or GFC. Its basic principle is the separation of molecules according to size.

Generally, a special gel for separation and a series of known molecular weight polysaccharides as standards are necessary for calculating the molecular weight of the goal polysaccharide, which depend on the calibration curve of the molecular weight and elution volume [80-99]. However, a major drawback of SEC is the difficulty to obtain suitable standards that possess the same hydrodynamic volume as the samples in the solution for calibration curve. Combined with laser light scattering (LLS), SEC is conveniently applied for determination of the molecular mass, molecular mass distribution and chain conformation of polymers without the aid of standard samples [100-102]. In recent years, SEC-LLS in combination with concentration detectors such as UV and RI has been demonstrated to be a very powerful method for characterization and analysis of polysaccharides from medicinal plants and fungus [103-106], which has superior accuracy of molar-mass determination because the molar masses of polysaccharide estimated based on the secondary calibration of standard dextrans is dependent upon initial assumptions about the behavior of the polysaccharide fragments relative to standard compounds [107-109].

In addition, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has proven to be an effective tool for the analysis of oligo- and polysaccharides in last decade [110,111]. In combination with SEC, MALDI-MS has been employed to obtain the average molar masses for polysaccharides [112,113]. Besides SEC, field-flow fractionation (FFF) is a separation method suitable for the characterization of macromolecules. Flow FFF connected to multi-angle light scattering detection (MALS) may be a successful way to obtain molecular masses and distributions [114-117]. The methods for measuring molecular weight of polysaccharides were shown in Table 2.

3.3. The Types and Ratios of Constituent Monosaccharides

Polysaccharide is a complex of different monosaccharides. Therefore, the types and ratios of constituent monosaccharides are the basic and important structural characteristics of polysaccharides. It was reported that the anticomplementary activity of fucan enhanced with the increased contents of galactose and glucuronic acid, which suggested that these residues should be essential [142].

Multiple linear regression analysis was used to deduce the correlation between the monosaccharide composition ratios of polysaccharides isolated from 10 regionally different strains of *Lentinula edodes* and their *in vitro* macrophage stimulatory activities [143]. The results showed that the compositions of arabinose, xylose, mannose and galactose were important. Especially, glucose, although presented in large compositions in all strains presumably forms the backbone of the polysaccharide structures, is not the determinant factor for either structural characteristics or that of the *in vitro* macrophage stimulatory activities. The study offered a potential method for the elucidation of polysaccharide structures and biological activities.

The types and molar ratios of constituent monosaccharides of polysaccharides are usually determined using PC, TLC, GC, HPLC and CE methods. Prior to chromatographic separation, acid hydrolysis (e.g. sulfuric acid, hydrochloric acid, or trifluoroacetatic acid i.e. TFA) of polysaccharides and followed neutralization with sodium hydroxide, barium

hydroxide or barium carbonate is necessary to obtain the constituent monosaccharides. Then the hydrolysate could be directly analyzed using TLC or PC with the help of standard monosaccharides analyzed at the same conditions. But they have low sensitivity and accuracy [32,84,88,96,97,144-146].

Gas chromatography (GC) is a unique and versatile technique, which is conventional method for analysis of volatile compounds. If the sample to be analyzed is nonvolatile, the techniques of derivatization or pyrolysis GC should be utilized. Up to date, GC has been widely applied to determine free monosaccharides, and constituent monosaccharides of both oligosaccharides and polysaccharides, which has the advantages of simple instrumentation, high selectivity and high accuracy. The derivatives could be prepared using monosaccharides with hexamethyldisilane (HMDS), trimethylchlorosilane (TMCS), trimethylsilyl (TMS), N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), etc. in nonaqueous organic solvents such as pyridine or dimethyl sulfoxide to obtain trimethylsilyl ether derivatives [147-150], or with hydroxylamine hydrochloride and acetic anhydride to form acetate derivatives in pyridine, butylenes oxide or methyl imidazole solvents [151-155]. FID is the commonly used detection for GC analysis, which has better selectivity and higher sensitivity. But MS could offer more reliable structure information for identification. The selectivity and accuracy are also greatly improved with the help of extracted ion count [156-164].

HPLC is also extensively applied for determination of constituent monosaccharides. In most cases, HPLC with ultraviolet (UV) detection is the prevailing technique, which has been widely used for determination of components in Chinese medicine. However, monosaccharides have no UV absorptivity. To detect intact monosaccharides, RI detection has been used for analysis by HPLC [165,167]. Unfortunately, RI detector is one of the least sensitive LC detectors, and it can not be used for gradient elution. Fluorescence derivatives of monosaccharides could be prepared to improve the sensitivity of analysis [67]. But derivation increases the complexity of sample preparation. The evaporative light scattering detector (ELSD) response does not depend on the samples' optical characteristics, which eliminates the problems associated with RI detector. Therefore, ELSD is increasingly being used in liquid chromatography as a quasi-universal detector, which has been successfully applied to analyze the compounds less volatile than the mobile phase, such as carbohydrates [166-168]. It is valuable to develop HPLC-ELSD method for direct analysis of carbohydrates, including monosaccharides, oligosaccharides and polysaccharides. Furthermore, polysaccharide analysis using carbohydrate gel electrophoresis (PACE) is a fast and simple technique for sugar composition analysis relies on derivatization of reducing ends of sugars with fluorophore [169,170], and as little as 500 fmol monosaccharides could be detected that showed the method is more sensitive [171]. In additional, CE has been developed as an attractive analytical method owing to its high separation efficiency, low sample consumption, short analysis time and relatively simple instrumentation. While electrochemical detection (ED) possesses higher sensitivity and lower detection limit than UV absorption. Hence, the CE-ED method is successfully used in the analysis of monosaccharides [172,173]. Table 3 listed the applications of major chromatographic methods for analysis of monosaccharides in some medicinal plants and fungi.

3.4. The Features of Glycosidic Linkages

The primary structure of a polysaccharide is defined by monosaccharide composition, configuration of glycosidic linkages, position of glycosidic linkages, sequence of monosaccharides, as well as the nature, number and location of appended non-carbohydrate groups. It is known that the position and configuration of glycosidic linkages have close relationship with their biological activities [198,199]. Unlike protein, there are more potential linkages in the repeated unit of polysaccharide. The multiplicity of possible linkages between monomeric units adds a further layer of structural complexity to carbohydrates because they may be linked between the anomeric hydroxyl group of one monosaccharide and any other hydroxyl-bearing carbon in another monosaccharide. The wide variety of positional and anomeric structures makes it possible for saccharides to form as many as 10^{12} distinct structures from as few as six different monosaccharide units [200]. The positions of glycosidic linkages can be analyzed by enzyme digestion, methylation analysis and NMR spectroscopy (Table 4). The last two are main methods because exoglycosidic digestion is limited to a few enzymes of high specificity.

In methylation analysis, polysaccharides should be sequentially methylated, hydrolyzed, reduced, and acetylated to form partially methylated alditol acetates which separated and analyzed using GC-MS. Various methylation methods have been developed and the dimsyl anion or alkali-metal hydroxide (e.g., NaOH) is typically employed to deprotonate free hydroxyls on the saccharide prior to methylation. Dimethyl sulfoxide (DMSO) is a commonly used solvent for the methylation reaction due to the good solubility of many saccharide species in this anhydrous solvent. However, solubility of high-molecular weight (HMW) polysaccharides is limited in DMSO, and often these polysaccharides are chemically or enzymatically degraded prior to methylation and linkage analysis. Glycerol could also be used for improving solubilization of HMW polysaccharides in methylation and linkage analysis [201]. Resulting chromatographic peaks are identified by a combination of their retention times and their electron impact-mass spectrometry (EI-MS) fragmentation patterns. In this way, which residues are terminal and how each monosaccharide is substituted are indicated, and the occurrence of branching points could also be identified [33,35,36,76,83,86,89,90,93,98,101, 107,159].

However, methylation analysis does not provide information on the sequence of constituent residues and the configuration of glycosidic linkages. NMR analysis is a powerful method for the structural analysis of polysaccharides. It is a fast, reliable, and nondestructive technique.

Table 3. The methods for determination of constituent monosaccharides in polysaccharides

Techniques	Methods			Ref.
	Sample preparation	Column	Detection	
GC				
	Hydrolysis: TFA, H_2SO_4, HCl–MeOH or methanolysis Derivatization: alditol acetates, acetylated aldononitriles or trimethylsilylated (TMS) derivatives	BPX-70, CP-Sil 5 CP, DB-series, HP-5, HP-Ultra2, OV-series, RTX-1, SE- series, Supelo SPB-1, Supelco SP series, 3% ECNSS-M and 1% OV-225 packed column or 3% SP 2340 and 3% OV-17 paked column	FID or MS	20-25, 28, 30, 36, 38, 41-43, 46-54, 56, 58-61, 63-66, 83, 100, 118, 119, 121, 122, 125, 126, 128, 129, 133, 136, 137, 139-141, 156, 158, 162, 174-189
HPLC				
	Hydrolysis: TFA, H_2SO_4 or HF and TFA Derivatization: labeled with 2-AB (2-aminobenzamide), labeled with PMP (1-phenyl-3-methyl-5-pyrazolone)	Alltima C18, CarboSep CHO-682, CarboPac PA1, TSKgel ODS120T, Dionex DX500, Nova-Packamino, TSKgel SUGAR AXI or Zorbax extend-C18	RI, UV, PAD or Fluorescence	44, 67, 104, 120, 123, 128, 131, 132, 165, 167, 190-195
CE				
	Hydrolysis: TFA	Uncoated fused-silica capillaries	UV	196
TLC and PC				
	Hydrolysis: TFA or H_2SO_4,	Precoated PEI-cellulose, Silica G gel or Slurry made of silica gel, Whatman Nos. 1 and 3 mm papers or Xinhua No. 1 paper	Aniline–O-phthalic acid, Aniline/Dipheny-lamine/phosphoric acid, Phthalic acid, Alkaline silver nitrate solution, Aniline hydrogen phathalate or 1,3-naphthalenediol reagent	24, 43, 45, 47, 48, 50, 54, 59-62, 64, 83, 156, 158, 178, 182, 184
NMR				
	D_2O solution	-	-	197

GC, Gas chromatography; **HPLC,** High performance liquid chromatography; **CE** Capillary electrophoresis; **TIC,** Thin layer chromatography; **PC,** Paper chromatography; **NMR,** Nuclear magnetic resonance; **FID**, Flame ionisation detector; **MS**, Mass spectrometery; **RI**, Refractive index; **PAD**, Pulsed amperometric detector; **UV,** Ultraviolet; **HF**, Hydrofluoric acid; **TFA**, Trifluoroacetic acid; **D_2O,** Heavy water.

Table 4. The methods for analysis of glycosidic linkage in polysaccharides

Techniques	Methods			Ref.
	Sample preparation	Column	Detection	
GC				
	Hydrolysis: TFA, H_2SO_4, MeOH–HCl or Formic acid Derivatization: partially methylated aditol acetates	BPX-series, CP-Sil 5 CP, DB-series, HP-series, OV-series, SPB-1, Supelco SP series column or 3% ECNSS-M and 1% OV-225 paked column	FID or MS	24, 25, 28, 34, 36, 40-43, 45, 46, 48-51, 53-60, 62, 63, 65, 83, 100, 119, 120, 122, 131, 136, 158, 185, 162, 165, 167, 174, 176, 178-180, 182-184, 186-189, 212
HPLC				
	Treated by galactanase and RG-hydrolase	TSKgel series	MALDI-TOF MS	194
IR				
	Powdered samples were dispersed in KBr pellets	-	-	213
NMR				
	D_2O solution	-	-	20-22, 40, 41, 47-52, 54, 56-59, 104, 120-122, 141, 165, 174, 176, 183, 204
	DMSO-δ6 solution	-	-	179

GC, Gas chromatography; **HPLC,** High performance liquid chromatography; **IR**, Infrared spectroscopy; **NMR,** Nuclear magnetic resonance; **FID**, Flame ionisation detector; **MS**, Mass spectrometery; **TFA**, Trifluoroacetic acid; **D_2O,** Heavy water; **DMSO,** Dimethylsulfoxide; **MALDI-TOF MS**, Matrix-assisted laser desorption/ionization-time of flight mass spectrometery.

Several NMR spectroscopic methods have been developed that permit the determination of linkage positions in oligosaccharides [202,203]. The altered chemical shift (δ) also indicated the configuration of glycosidic linkages, and their ratio could be calculated based on their relative peak area. NMR with [204-206] or without [203] assistance of other methods has been applied for elucidation of anomeric configurations. In addition, MS has become one of the most powerful and versatile techniques for structural analysis of carbohydrates [207-209]. The most widely used mass spectrometric approach is electrospray ionization (ESI) tandem-MS, typically performed on triple-quadrupole instruments using precursor-ion selection in a first MS step, collision-induced dissociation and mass analysis of fragment ions in a second MS step [210]. However, ESI are not widely used for direct analysis of neutral glycans due to the ionization is poor. Thus, the derivatization is usually applied prior to ESI-MS characterization with the aim of increasing analytical performance and sensitivity [211].

3.5. Fingerprint

It is complex, difficult and time consumable though structural information is unambiguous identification of polysaccharides. Therefore, how to discriminate the polysaccharides from different origins is crucial for quality control of polysaccharides. In last decade, the profiling of the relative amounts of various active ingredients (i.e. fingerprint profiling) has been shown to be a convenient and effective method for the quality control of various herbal materials, especially when there is a lack of authentic standards for the identification of all the active components present in these complex natural products [214-217]. This technique is also successfully applied for the identification and characterization of protein [218-222]. However, there are few reports for quality control of carbohydrates based on their fingerprints. This is perhaps not surprising since the separation and detection of carbohydrates, especially long-chain polysaccharides, is well known to be a highly challenging and difficult analytical problem [223,224]. The sugar profiles of extracellular polysaccharides, which were derivatised to alditol acetates and identified by GC, from different *Tremella* species showed that all of the polysaccharides contained essentially the same sugars but in different ratios [225]. The high-performance thin-layer chromatography (HPTLC) peak profiles of acid hydrolyzates of carbohydrates from various Lingzhi species/products were also demonstrated. The unique fingerprint patterns were observed in the monosaccharide profiles between two highly valued Lingzhi species, *Ganoderma applanatum* and *Ganoderma lucidum*, under total or partial acid hydrolysis conditions [226].

The three *Angelica* polysaccharides fractions were identified using HPLC after hydrolysis and subsequently labeled with 1-phynyl-3-methyl-5- pyrazolone [195]. However, the extent of acid hydrolysis is difficult to control and some monosaccharide could be degraded under the acid conditions [227-229]. The ratio of monosaccharides obtained in acid hydrolysate may be not in accordance with that in polysaccharides. Therefore, highly specific enzymatic digestion of polysaccharides was developed, and a unique fingerprint of short oligosaccharides was produced because the oligosaccharides of identical mass but different monosaccharide composition do not co-migrate in the gels [230]. The oligosaccharides released by enzyme hydrolysis were derivatised with a fluorophore at their reducing end, and then separated by PACE [231] or CE [232]. The structural isomers of partially methylesterified oligogalacturonides also can easily be separated and quantified using PACE [233]. CE coupled with [234] or without [235] MS has also been widely applied for analysis of polysaccharides. Furthermore, high performance GPC (HPGPC) was developed for quality control of polysaccharides in natural and cultured *Cordyceps* [236], as well as Lingzhi product [237]. Its application on analysis of enzymatically treated cellulose and related polysaccharides has also been reviewed [238]. Lipid profile in meningococcal polysaccharide was also determined using reversed-phase liquid chromatography. The capsular meningococcal polysaccharide (MnPs) of *Neisseria meningitidis* is an antigenic component, which is comprised of only even-chain fatty acids. Its purification must remove endotoxin, a pyrogenic lipopolysaccharide impurity, which differs that the C_{12} and C_{14} fatty acids are hydroxylated in the gamma position. Consequently, a fast and sensitive HPLC method using fluorescence detection differentiates fatty acids provides an assay for both of these lipids,

which was used against process intermediate samples to assist the purification development [239].

4. Quantitative Analysis of Polysaccharides

Quantitation is very important for quality control of polysaccharides. However, compared to monosaccharides and oligosaccharides, the analysis of polysaccharides is more difficult due to their large molecular weights, complex structures and inert chemical activations. Colorimetry such as phenol–sulfuric acid, anthracenone–sulfuric acid and carbazole–sulfuric acid, which take use of the constituent monosaccharides properties, are commonly used for quantitative determination of total polysaccharides due to their simplicity and economy [61,94,97]. Among these methods, the phenol-sulfuric acid assay is the most frequently used. But the reproducible test results greatly depend on the modality of acid addition [241,242], which can be improved by means of two modifications [241]. One is a slow, careful acid addition over the side of the tube, with incubation at 110 ^{o}C. The other is using a model mixture as the standard, made up of the individual sugars of the polysaccharide in the same molar ratio as in the natural substance. Even though the procedure is modified, the selectivity and sensitivity are still unsatisfactory when quantifying certain deoxy- and amino-sugar derivatives. In such cases, the Morgan-Elson assay can be performed, where N-acetyl- or amino-sugars are heated in an alkaline solution to form a chromogen, which produces a purple colored compound when reacted with N,N-dimethyl-p-aminobenzaldehyde in an acid solution. The absorbance is determined using a spectrophotometer at the appropriate wavelength of 530 nm for amino-sugars, and either 544 nm or 585 nm for N-acetyl-sugars. The measurement of uronic acid content was determined according to a m-hydroxydiphenyl colorimetric method in which neutral sugars do not interfere [61,94,97,243]. In general, colorimetry for quantitation of polysaccharides is simple and rapid, but the selectivity and sensitivity are poor. Therefore, separation techniques, including chromatographic and electromigratic methods, with appropriate detection (e.g. UV, ED, RI and MS, etc.) are greatly increasing on application for quantitative analysis of polysaccharides [5] (Table 5).

In most cases, the constituent monosaccharides are considered as the targets for quantitation of polysaccharides because appropriate reference polysaccharides are difficult to obtain. Thus hydrolysis followed with or without derivation is necessary before quantitative analysis, which has been introduced in *Section 3.5*. Considering the degradation of certain monosaccharide residues under acid hydrolysis, enzyme hydrolysis has also been employed [244-248]. GC-FID (flame ionisation detector) [249], GC-MS [250], HPLC-UV [251], HPLC-RI [227], high-performance ionic chromatography coupled to pulse amperometry detection (HPIC-PAD) [252-256], and LC-MS/MS [257] are widely used for separation and detection so as to quantitate the content of polysaccharides in different matrixs. In addition, the other constituent components such as uronic acid could also be used for quantitative analysis of polysaccharides [258]. Especially, by highly specific enzymatic digestion of a polysaccharide in a cell wall preparation, a unique short oligosaccharide which gave quantitative and structural information on the original polysaccharide chain was produced.

The quantity of digested polysaccharide measured using the optimized protocols was very reproducible, but it may not represent the entire amount of structurally related polysaccharide in the cell wall materials because the substitutions of the polysaccharide can prevent digestion by the enzymes and a fraction of the polysaccharide might remain undigestible [230]. On the other hand, though there are a few reports for direct quantitative determination of polysaccharides [259-262], quantitative analysis of individual polysaccharides remains challenging.

Table 5. A summary for quantitative analysis of polysaccharides from different origins

Origins	Targets	Methods					Ref.
		Techni-ques	Sample preparation	Column	Detec-tion	LOD	
Vegetables	Monosacc-hairdes	GC	Hydrolysis: ①12M H_2SO_4, 35℃, 30min; then ②2M H_2SO_4, 100℃, 1h Derivatization: alditol acetate	SP-2330 column (30 m × 0.25 mm × 0.25 μm)	FID	Not mentioned	227
		HPLC	Hydrolysis: ①12M H_2SO_4, 40℃, 1h; then ②0.4M H_2SO_4, 100℃, 3h	Aminex HPX-87P (30 cm × 7.8 mm, I.D.)	RI	Not mentioned	
Dextran	Disaccha-rides	LC-MS	①Enzymatically hydrolysisi: α-1,6-glucosidase (dextranase); then ② acetylation of the generated isomaltose subunits	Zorbax SB-C18 (15 cm × 4.6 mm × 3.5 μm)	MS/ MS	3.8 μg/mL	245
Streptococcus pneumoniae	Capsular polysacch-aride	Colori-metry	Modified phenol–sulfuric acid method	-	Vis 490 nm	Not mentioned	242
	Monosacc-harides	GC-MS	Hydrolysis: methanolic 3N HCl, 121℃, 2h Derivatization: trimethylsilyl (TMS) derivative	HP-5 capillary	MS	10 ng	250
Streptococcus pneumoniae	Monosacc-harides	HPLC	Hydrolysis: 2M TFA, 121℃, 2h; or ①2M HF in Methanol, 80℃, 24h; then ②2M TFA ,121℃, 2h	Carbopac PA10 analytical (25 cm × 4 mm, I.D.)	PAD	Not mentioned	252
			Hydrolysis: ①48% HF, 65℃, 2 h; then ② 2M TFA , 120℃, 2 h	MA1 analytical	PAD	Not mentioned	253
Streptococcus pneumoniae	Monosacc-harides	HPLC	Hydrolysis: 2M TFA, 121℃, 2h; or ①2M HF in Methanol, 80℃, 24h; then ②2M TFA ,121℃, 2h	Carbopac PA10 analytical (25 cm × 4 mm, I.D.)	PAD	Not mentioned	252

Table 5. (Continued)

Origins	Targets	Methods					Ref.
		Techniques	Sample preparation	Column	Detection	LOD	
Neisseria meningitidis serogroup A	Monosaccharides	HPLC	Hydrolysis: 2M TFA, 80℃, 3h	CarboPac PA1 IonPac AS11 (25 cm × 4 mm, I.D.)	PAD	Not mentioned	255
Saccharomyces cerevisiae	Monosaccharides	HPLC	Hydrolysis: 72% H_2SO_4, room temperature, 3 h	CarboPac PA1 anion-exchange (25 cm × 4mm, I.D.)	PAD	Not mentioned	256
Cationic polysaccharides	Monosaccharides	LC-MS	Hydrolysis: 2M TFA, 100℃, 2h	Bore column (60 mm×2 mm, I.D.) packed with GromSil Diol stationary phase	MS	5.4 μg (LOQ)	257
Uronic acid	D-glucuronic acid, D-galacturonic acid, D-mannuronic acid	HPLC	Derivatization: postcolumn fluorescence with benzamidine	Whatman Partisil-10SAX (25 cm × 4.6 mm, I.D.)	UV 210 nm	7.91 pmol 23.88 pmol 7.08 pmol	258
Chitosan	Chitosan	CE	Dissolved in 100 mM triethylamine (TEA)-phosphate buffer, pH 2.0	Untreated fused-silica capillary (27 cm × 50 μm, I.D.) Neutral coated capillary (27 cm × 50 μm, I.D.)	DAD	0.25 mg/mL	259
Neisseria meningiditis serogroups A, C, W135, Y	Meningococcal polysaccharides	CE	Dissolved in water	Uncoated fused silica capillary (30 cm × 50 μm, I.D.)	UV	Not mentioned	260
Food products	Gellan gum	CE CE-MS	Digestion by gellan-lyase	Hewlett packard MS fused silica capillary (80 cm × 50 μm, I.D.)	DAD ESI-MS	Not mentioned	261
Dermatan Sulfate (DS) Oversulfated DS	DS, oversulfated DS	HPLC	Derivatization: React with guanidine in 0.5M NaOH, 110℃	Asahipak NH_2P-50 (25cm × 4.6 mm, I.D.)	Fluorescence	10 ng 20 ng	262
Pseudomonas aeruginosa, *Streptococcus mutans*, *Staphylococcus epidermidi*	Total sugar	IR	KBr beam	-	IR	Not mentioned	264

GC, Gas chromatography; **HPLC,** High performance liquid chromatography; **IR**, Infrared Spectroscopy; **CE**, Capillary chromatography; **LOD,** Limit of detection; **ESI-MS**, Electrospray ionization mass spectrometry; **LOQ,** Limit of quantification; **FID**, Flame ionization detector; **RI**, Refractive index; **DAD**, Diode array detector; **PAD**, Pulsed amperometric detector; **UV,** Ultraviolet; **TFA**, Trifluoroacetic acid; **HF**, Hydrofluoric acid.

5. Conclusion

In conclusion, quality control of polysaccharides is a challenge because of its complexity. The rapid, sensitive and selective methods for quanlitative and quantitative analysis of polysaccharides are still the research interest. The relationship between chemical characteristics and the biological activities of polysaccharides will also intensively attracte wide attention of pharmaceutical and biochemical analysts.

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Chapter 13

Medicinal Plants: A Tool to Overcome Antibiotic Resistance?

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Abstract

Bacterial antibiotic resistance has become a serious problem of public health that concerns almost all antibacterial agents and that manifests in all fields of their application. Consequently, there is an increasing interest in the search for new compounds which can act by a direct antimicrobial effect or by inhibiting resistance mechanisms of microorganisms of medical importance. Medicinal plants nowadays remain a valuable source for this kind of compounds. The direct antimicrobial properties of a number of natural compounds have indeed been reported; such compounds act by many mechanisms, including: *(i)* complexation with macromolecules such as proteins and polysaccharides, thus inhibiting their functions (polyphenols); *(ii)* disruption of microbial membranes (lipophilic flavonoids, terpenoids, plant defensins); and *(iii)* inhibition of adhesion of microbial proteins to host polysaccharide receptors (polypeptides). Medicinal plants also provide compounds which are not necessarily effective against microorganisms, but which enhance or restore the activity of antibiotics by inhibiting resistance mechanisms. These compounds belong to several phytochemical groups and act as inhibitors of efflux pumps (flavonoids, terpenoids, alkaloids); inhibitors of PBP 2a (quinones, terpenoids), enhancers of the permeability of bacterial membrane (terpenoids) and beta-lactamases inhibitors (alkyls gallates).

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1. Introduction

Infectious diseases caused by bacteria, fungi, viruses and parasites remain a major threat to public health due to the emergence of widespread antimicrobial resistance (WHO, 1996) increasing at an alarming rate (Hawkey, 2000). Thus, it is essential that resistance to currently used antimicrobial agents be prevented, limited or reversed. Antibiotic resistance is the ability of microorganisms to remain impervious to the inhibitory or lethal effect of antibiotics (Kaye et al., 2000). This resistance can be intrinsic, inherent to a particular species; for example gram-negative bacteria are intrinsically resistant to vancomycin because these organisms contain an additional protective outer membrane, absent in gram-positive cells, that prevents the agent from reaching the target site (Walsh, 2003). The resistance can also be acquired, when it refers to an attribute resulting from a change in the genetic composition of the bacteria, rendering a previously active drug ineffective (Rice et al., 2003).

If inappropriate prescribing and use of antibiotics are considered as the major factors in the emergence of the resistance phenomenon, education addressed to both prescribers and patients can help to reduce resistance (Yates, 1999). A further approach resides in the research for new drugs, acting by other mechanisms than those described for existing antibiotics, or inhibiting resistance mechanisms of microorganisms of medical importance. These new drugs can be provided by natural sources, particularly by medicinal plants. Indeed, medicinal plants have always provided modern therapeutic a most important source of lead compounds in the search of new drugs and medicines (Cowan, 1999). Indigenous herbals are widely used against many infectious diseases and can be a valuable source for natural compounds, with a great interest in the fight against pathogenic bacteria and antibiotics resistance. Many studies report the antimicrobial properties of secondary metabolites from medicinal plants, of which it is estimated that less than 10% of the total have been characterized (Schultes, 1978). Such compounds are part of "vegetal immunity", complex mechanisms of plants' defenses against predation by microorganisms, insects and herbivores. Interestingly, some phytochemical compounds, although lacking antimicrobial activity, display an inhibitory effect on resistance mechanisms of microorganisms, thus enhancing or restoring the activity of some antibiotics. All these compounds belong to many phytochemical groups found in several botanical families; .among these, tannins and essential oils are particularly known for their direct antimicrobial properties.

The antimicrobial activity of plant extracts, essential oils or pure compounds can be assessed by different methods, including broth dilution (determination of minimum inhibitory concentrations), diffusion on agar (determination of inhibition diameter) and bioautography (localization of active compounds on a TLC plate) (Botz et al., 2001). The solvents used for plant extraction are an important factor in the antimicrobial evaluation; there are solvents more favorable according to the number of inhibitors extracted (Vanden Berghe et Vlietinck, 1991; Eloff, 1998). The collection and conservation of plant materials is also important to ensure correct botanical identification and preservation of biological activities.

The present paper reviews *(i)* the different methods of evaluation of antimicrobial activity of plant extracts and natural compounds, *(ii)* the principal groups of direct

antimicrobial compounds from medicinal plants and their mechanisms of action; and *(iii)* the natural compounds inhibiting resistance mechanisms of microorganisms.

2. Extraction and Biological Tests of Medicinal Plants

2.1. Extraction

The screening of medicinal plants for antimicrobial activity follows a logical pathway. Plants are collected randomly or by following instructions given by traditional healers in the areas where the plants are found (Verpoorte et al., 2005). Any part of the plant may contain antimicrobial compounds, for instance, the roots of *Albertisia villosa* contain antimicrobial alkaloids (Lohombo-Ekomba et al., 2004), while eucalyptus leaves are harvested for their essential oils and tannins. It is also possible to use herbarium specimens to test the antimicrobial activity of medicinal plants (Eloff, 1999).

Crude aqueous or alcohol extracts are typically used for the preliminary antimicrobial tests; they can be followed by various organic extractions. For alcoholic extracts, plant parts are dried, ground to a fine texture, and then soaked in methanol or ethanol for extended periods. The slurry is filtered and the filtrate dried under reduced pressure. Crude extracts can then be used in agar disk diffusion or broth dilution tests for antimicrobial properties and for other biological activities. Active crude extracts can be submitted to a bio-guided fractionation in order to purify and identify the active compounds by various techniques including chromatography and spectroscopy (Pieters et Vlietinck, 2005).

Eloff has examined the ability to extract antimicrobial compounds from plants of a variety of solvents, as well as other factors such as their relative rankings as biohazards and the ease of removal from fractions (Eloff, 1998). Acetone, which is not one of the most frequently used extractants in published studies, received the highest overall rating, followed by dichloromethane, methanol, ethanol and water, respectively. This study suggests that most of the active antimicrobial compounds are not water-soluble and that the most commonly used solvents may not present the highest sensitivity in initial screenings for antimicrobial compounds.

2.2. Agar Diffusion Methods

The agar diffusion assay is one of the most commonly used methods for antimicrobials susceptibility testing. Test compounds at known concentrations are brought in contact with an inoculated agar medium, the inoculated system is maintained at room temperature for several hours in order to favor the diffusion of test compounds over the microbial growth surface. The system is then incubated at requested t° and the diameter of the clear zone around the disk of test compound ("inhibition diameter") is measured after the incubation period. The diffusion method is not appropriate for testing non-polar samples or samples that do not easily diffuse into agar. In general, the relative antimicrobial potency of different samples may not always be compared, mainly because of differences in physical properties, such as solubility, volatility and diffusion characteristics in agar (Cos et al., 2006).

The agar diffusion technique can also be used to study the effect of plant extracts on antibiotics resistance. In this purpose, inactive plant extracts are dissolved and incorporated in molten agar medium and cast in Petri dish (Okusa et al., 2007); suitable controls are prepared with medium without plant extracts. The susceptibility of antibiotics is then evaluated by a classical disk diffusion method. Another possible approach is the incorporation of antibiotic in the agar layer overlaid by disks containing tested plant extracts (Shahverdi et al., 2004).

2.3. Liquid-and Agar-Dilution Methods

Liquid broth dilution methods, including microdilution methods, are widely used to quantitatively measure the *in vitro* activity of an antimicrobial agent against a given bacterial strain. A series of serial dilutions of the tested agent in broth tubes or multiwell plates are inoculated with a standardized suspension of the test microorganism. After overnight incubation, the minimum inhibitory concentration (MIC) is visually or spectrometrically estimated by determination of turbidity (NCCLS, 2003) or of reaction products from redox-indicators such as MTT or resazurin (Gabrielson et al., 2002). Test samples may react with the chromogenic reagent or, if not fully soluble, may interfere with turbidity readings, emphasizing the need for suitable controls, i.e. extract dissolved in blank medium without micro-organisms. The liquid-dilution method also allows determination whether a compound or extract has a cidal or static action at a particular concentration. The minimal bactericidal or fungicidal concentration (MBC or MFC) is determined by plating-out samples of completely inhibited dilution cultures on agar medium and visually assessing growth (static, MIC) or no-growth (cidal, MBC) after incubation (NCCLS, 2002, 2003).

In agar-dilution methods, various concentrations of antibacterial substance are mixed with nutrient agar and caste. Casted agar plates are then inoculated and incubated. The lowest concentration of antimicrobial compound resulting in no visible growth is considered as the MIC value.

Liquid-and agar-dilution methods can also be used to study the effect of plant extracts on antibiotics resistance.

2.4. Synergy between Plant Extracts and Antibiotics

Antimicrobial combinations are considered to be synergistic if the effect of combination is greater than the effect of either agent alone or greater than the sum of the effects of individual agents. Antagonism results if the combination provides an effect less than the effect of either agent alone or less than the sum of the effects of the individual agents. Indifference results if the combination provides an effect equal to the effect of either agent alone. The distinction between synergy, antagonism and indifference classically relies on the determination of Fractional Inhibitory Concentrations (FIC= MIC of a drug given in combination/MIC of the same drug alone) and FIC index (for a mixture of drugs A and B, FIC index = FICA + FICB). The FIC index is classically evaluated as follows: synergy (FIC

index ≤ 0.5), additive (0.5 < FIC index ≤1), indifference (1 < FIC index ≤ 2) and antagonism (FIC index > 2) (Mackay et al., 2000).

2.5. Bioautography on Thin-Layer Chromatoplates

TLC-bioautography, a convenient and simple way of testing plant extracts and pure substances for their effects on pathogenic microorganisms, allows an easy detection of active fractions (Hostettmann et al., 1997). Three variants of the technique are described: *(i)* direct bioautography, in which a broth of the micro-organism is directly applied on the TLC plate; *(ii)* contact bio-autography, where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact; and *(iii)* agar-overlay bioautography, where a seeded agar medium is applied directly onto the TLC plate (Botz et al., 2001). In the 1960's, contact and immersion bioautography in various versions were routinely used, but nowadays direct bioautography largely prevails (Hamburger et Cordell, 1987).

TLC is usually performed in duplicate with suitable mobile phases and the plates are dried to remove the eluents, a critical step. One set of plates is used as the "reference" chromatogram, spots being visualized under UV light or by spraying a specific or a general reagent (Wagner et Bladt, 1996). The second plate is placed in a Petri dish, aseptically overlaid by the inoculated medium and incubated overnight. The bioautogram is subsequently sprayed with an aqueous solution of MTT 0.8 mg/ml and incubated for a few hours (Botz et al., 2001). The MTT is converted to a formazan dye by the microorganisms and active compounds are indicated by inhibition zones observed as clear spots against a purple background (Begue et Kline, 1972). The applicability of TLC-bioautography is however limited to microorganisms that easily grow on TLC plates and requires complete removal of residual low volatility solvents, such as *n*-BuOH, trifluoroacetic acid or ammonia, which can inhibit the growth of several microorganisms (Nagy et al., 2002).

2.6. Antiparasitic Test

In contast to antibacterial, antifungal and antiviral assays that are based on common test conditions and endpoints, antiparasitic assays are more complicated and more exclusive since they tend to be highly species-specific (*Plasmodium*, *Entamoeba*, helminthes...). Particular interest is devoted to malaria, one of the protozoal diseases that have been defined by the WHO as major health risks. Extracts possibly effective against *Plasmodium* can be tested on microorganisms in an erythrocyte infection assay on microtiter plates. Parasitized erythrocytes are incubated with and without test substances, and the numbers of *Plasmodium* organisms after the incubation period are quantitated either by measurements of radioactivity (radiolabeleld parasites) (Cos et al., 2006) or of the parasite lactate deshydrogenase (pLDH) activity, using 3-acetyl pyridine NAD (APAD) as a coenzyme; as human red blood cell LDH carries out this reaction at a very slow rate in the presence of APAD, the measurements correlate with the levels of parasitemia (Makler et al., 1993).

2.7. General Toxicity Tests

To assess the selectivity of the observed antimicrobial activity, cytotoxicity assays on mammalian cells is very important and should be included in parallel. A MRC-5 cells model is frequently used; cell proliferation and viability is assessed either by visual counting or by spectrophotometry after addition of MTT, Alamar BlueTM or resazurin (McMillian et al., 2002).

3. Plant Compounds with Direct Antimicrobial Activity

Natural products from plants are a source of "lead" compounds in the search for new antimicrobial drugs and medicines. Over the past three decades, researchers have also turned to many of the traditional folk medicines, essentially cocktails of natural products, to uncover the scientific basis of their remedial effects, endeavors which have their roots in a desire to improve the efficacy of modern medical practice (Haslam, 1996). In this context, particular attention has been given to the traditional herbal medicines which provide antimicrobial compounds with newly discovered mechanisms of action. Table 1 lists examples of some antimicrobial compounds from medicinal plants.

3.1. Mechanisms of Direct Antimicrobial Action

3.1.1. Association with Macromolecules

The antimicrobial properties of some phytochemicals, notably polyphenols, may be attributed to their ability to form complexes with macromolecules such as proteins and polysaccharides. This propensity to bind proteins can presumably lead to an inhibition of enzymes. Assessment of the medical significance of inhibition of a particular enzyme, determined *in vitro*, is however dependant on the absorption and distribution of administered compounds to the desired *in vivo* site of action. Such limitations do not arise on local applications, especially when the enzymes are extracellular. Polyphenols for example are known to bind glycosyl transferases, enzymes involved in the synthesis of water-insoluble glucans from sucrose by *Streptococcus* mutans (Hattori et al., 1990). These glucans firmly bind the bacteria to the tooth surface, leading eventually to the formation of dental plaque and development of dental caries; polyphenols extracted from green tea showed inhibitory activity against glycosyl transferases and their administration led to highly significant reduction in dental caries in animals (Ooshima et al., 1993).

Antimicrobial quinones also often act by protein binding mechanisms. Indeed, quinones are known to covalently and irreversibly bind nucleophilic amino acids in proteins, often leading to inactivation of proteins (Cowan, 1999). Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes.

Table 1. Examples of some antimicrobial compounds from medicinal plants by class, source and activity

Compounds	Medicinal plants	Antimicrobial property			References
		Activity	MO highly sensitive	MIC range (µg/ml)	
Phenylpropanoids					
Eugenol	*Syzygium aromaticum (Myrtaceae)*	Antibacterial	*B.s, E.c, H.p*	2	(Ali et al., 2005; Rastogi et al., 2008)
Caffeic acid	*Olea europaea (Oleaceae)*	Antibacterial	*S.m, E.cl*	2	(Almeida et al., 2006; Pereira et al., 2007)
Flavonoids					
Licochalcone	*Glycyrrhiza inflate (Fabaceae)*	Antibacterial Antimucobacterial Antileishmanial Antiplasmodial Antiviral	*S.a, B.s, M.t* *L.m* *P.f* *H.I.V*	2 - 8 7 1.6 µM - -	(Shea et al., 1993; Chen et al., 1994; Zhai et al., 1995; Haraguchi et al., 1998; Friis-Moller et al., 2002; Tsukiyama et al., 2002)
Laburnetin	*Ficus chlamydocarpa (Moraceae)*	Antibacterial Antimucobacterial Antifungal	*S.a* *M. s* *C.a*	19 0.6 39	(Kuete et al., 2008)
Piliostigmol	*Piliostigma reticulatum (Caesalpiniaceae)*	Antibacterial Antifungal	*S.a, E.c* *C.a*	2 - 3 10	(Babajide et al., 2008)
Glabranin	*Helichrysum forskahlii (Asteraceae)*	Antibacterial	*S.a, B.s*	3 - 6	(Al-Rehaily et al., 2008)
Sulcatone A	*Ouratea sulcata (Ochnaceae)*	Antibacterial	*S.a, B.s*	8 - 12	(Pegnyemb et al., 2005)
Tannins					
Corilagin	*Cunonia macrophylla (Cunoniaceae)*	Antibacterial Antifungal	*S.a* *C.a*	- -	(Fogliani et al., 2005)
Quinones					
Newbouldiaquinone A	*Newbouldia laevis (Bignoniaceae)*	Antibacterial Antifungal Antimaplasmodial	*E.c, K.p* *C.g* *P.f*	0.3-10 0.31 -	(Eyong et al., 2005) (Kuete et al., 2007)
Zenkequinone B	*Stereospermum zenkeri (Bignoniaceae)*	Antibacterial	*P.a, E.c,*	9-18	(Lenta et al., 2007)
2-methyl-6-(-3-methyl-2-butenyl)benzo-1,4-quinone	*Gunnera perpensa (Haloragaceae)*	Antibacterial Antifungal	*B.c, S.a* *C.a*	18-39 130	(Buwa et van Staden, 2006) (Drewes et al., 2005)
Terpenoids					
Aethiopinone	*Salvia sclarea (Lamiaceae)*	Antibacterial	*S.a, S.e*	37-75	(Kuzma et al., 2007)

Table 1. (Continued)

Compounds	Medicinal plants	Antimicrobial property			References
		Activity	MO highly sensitive	MIC range (µg/ml)	
ent-beyer-15-en-19-ol	*Helichrysum tenax (Asteraceae)*	Antibacterial Antifungal	*B.c, S.e* *C.a*	3-31 62	(Drewes et al., 2006)
imberbic acid	*Combretum imberbe (Combrataceae)*	Antibacterial Antimucobacterial Antifungal	*S.a* *M.f* *C.a*	3 1.5 100	(Katerere et al., 2003; Angeh et al., 2007)
11-hydroxy-12-oxo-7,9(ll),13-abietatriene	*Plectranthus elegans (Lamiaceae)*	Antibacterial	*S.a, B.S*	10-40	(Dellar et al., 1996)
Epidioxysterol	*Morinda citrifolia*	Antibacterial Antimucobacterial Antifungal	*S.a, P.a* *M.t* *F.o*	- 2.5 -	(Levand et Larson, 1979; Saludes et al., 2002)
serrulatic acid	*Eremophila sturtii (Myoporaceae)*	Antibacterial	*S.a*	15	(Liu et al., 2006)
Alkaloids					
Cocsoline	*Albertisia villosa (Menispermaceae)*	Antibacterial Antifungal Antiparasitic	*S.t, E.c, S.s* *C.a* *Pf*	16-32 32 0.55µM	(Otshudi et al., 2005)
Cryptolepine	*Cryptolepis sanguinolenta (Periplocaceae)*	Antibacterial Antimucobacterial	*Cs* *Mt, Mf*	12 12-16	(Sawer et al., 1995; Cimanga et al., 1996; Paulo et al., 2000; Gibbons et al., 2003)
condaline A	*Condalia buxifolia (Rhamnaceae)*	Antibacterial	*K.p, E.c*	3 – 6	(Morel et al., 2002)
8-acetonyldihydronitidine	*Zanthoxylum tetraspermum*	Antibacterial Antifungal	*S.a* *C.a*	1.56 -	(Nissanka et al., 2001)
Plant defensins					
RsAFP2	*Raphanus sativus (Brassicaceae)*	Antibacterial Antifungal	 *F.s*	- 0.2 µM	(Terras et al., 1992; Thevissen et al., 1996; Gutierrez et Perez, 2004)
Cn-AMP1	*Cocos nucifera (Arecaceae)*	Antibacterial	*Sa, Pa*	80	(Mandal et al., 2008)
Cy-AMP1	*Cycas revoluta (Cycadaceae)*	Antibacterial Antifungal	*C.m* *F.o*	7.3 6	(Yokoyama et al., 2008)

Legend:MO: microorganisms, MIC: mimimum inhibitory concentration, S.a: Staphylococcus aureus, B.s: Bacillus subtilis, E.c: Escherichia coli, C.a: Candida albicans, P.s: Pseudomonas aeruginosa, L.m : Leishmania major, M.s : Mucobacterium smegmatis, M.t : Mucobacterium tuberculosis, C.g : Candida gabrata, K.p : Klebsiella pneumoniae, F.s : Fusarium solani, F.o : Fusarium oxysporum, C.m : Clavibacterium michiganensis, S.m : Serratia marcescens, E.cl :Enterobacter cloacae, H.p : Helicobacter pylori

3.1.2. Detergent-Like Membrane Disruption

Microbial membrane disruption can occur either by formation of pores which increase the membrane permeability to ions and larger molecules, or by hydrolysis of membrane phospholipids. Most of these bactericidal agents are peptides or peptide-based molecules that have been either isolated from natural sources or synthetically designed. Lipophilic flavonoids and terpenoids may also disrupt the microbial membranes.

As resistances to antimicrobial compounds that disrupt the structure of the bacterial membranes, rather than inhibiting a specific enzyme, are less likely, such compounds are considered as the probable future of antibiotics (Lockwood et Mayo, 2003).

3.1.3. Other Mechanisms

Since microorganisms need to adhere to host cells to cause infection, compounds that can inhibit the adhesion of microbial proteins to host polysaccharide receptors are potential antimicrobial agents. This mode of action is particularly encountered in lectin molecules and it is worth emphasizing that such antimicrobial lectins are not detected by using classical general antimicrobial screening protocols.

The antimicrobial and/or antiparasitic activity of planar molecules, including quaternary alkaloids (berberine, harmane) is probably due in part to their ability to intercalate within DNA (Omulokoli et al., 1997).

3.2. Phytochemical Classes of Antimicrobial Compounds

3.2.1. Antibiotic Compounds from Microorganisms

Antibiotics, agents "against life", can either be natural products or synthetic chemicals, designed to block some crucial process in microbial cells selectively. Most of the antibiotics introduced into human clinical use to treat infectious disease in the past 60 years have been natural products, elaborated by one microorganism in a particular habitat and set of environmental conditions to affect neighboring microbes, either to regulate their growth or to trigger their elimination (Walsh, 2003). Diverse structures isolated from a series of microorganisms have yielded the antibiotics, an extremely important class of medicinal compounds reviewed in details in many papers. Streptomycetes, gram-positive filamentous bacteria, account for the production of about 55 % of the commercially significant antibiotics including macrolides, glycopeptides, tetracyclines, β-lactams, aminoglycosides. Platensimycin, a previously unknown class of antibiotics, is produced by *Streptomyces platensis* and shows a strong, broad-spectrum Gram-positive antibacterial activity by selectively inhibiting cellular lipid biosynthesis (Wang et al., 2006).

3.2.2. Phenolic Compounds

Among the simplest bioactive phytochemicals, ***simple phenols and phenolic acids*** consist of a single, mono- or polysubstituted, phenolic ring and include compounds with interesting antimicrobial activity. For example, caffeic acid is effective against viruses, bacteria and fungi, catechol, pyrogallol and arbutine are shown to be toxic to microorganisms; the site and number of hydroxyl groups are thought to be related to the relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. The phenolic toxicity to microorganisms can be attributed to their oxidized, eventually quinone, forms which lead to enzyme inhibition, possibly through reaction with sulfhydryl and amino groups or through more non-specific interactions with proteins (Haslam, 1996). The phenylpropanoids (C6-C3), phenolic compounds with a C3 side chain, are found in essential oils and often cited as antimicrobial agents. Eugenol, a C6-C3

compound, present in clove oil, is effective against bacteria and fungi and has been widely used in dentistry.

Flavonoid and stilbene, structures based on a phenylchromane or a diphenylethene, are well-known phytoalexins, compounds synthesized by plants in response to microbial and fungal infections. It should not be surprising that they have been found to be effective against a wide array of microorganisms. The antimicrobial activity of teas is related to their content in catechins, reduced form of the C3 unit of flavonoids. These compounds are effective against many microorganisms including *Vibrio cholerae*, *Streptococcus mutans*, *Shigella sp*. Flavonoids are also effective against many viruses; swertifrancheside and chrysin have been found active against HIV; other flavone derivatives show an inhibitory effect on respiratory syncytial virus (RSV) (Cushnie et Lamb, 2005). Resveratrol, a diphenylethene phytoalexin commonly found in food and drinks, including red wine, grapes, and peanuts; is effective against bacteria and fungi.

Tannins, polymeric phenolic substances, characterized by their astringency, are capable of tanning leather or precipitating gelatin from solution. They are divided into three groups, *(i)* the hydrolysable tannins, based on gallic and ellagic acids usually as multiple esters with polyols; *(ii)* the procyanidols or condensed tannins which are oligo- and polymerized flavonoid monomers; and *(iii)* the complex tannins, comprising monomers from the other 2 classes. Many physiological activities, such as stimulation of phagocytic cells, host-mediated antitumor activity and antimicrobial effects, have been assigned to tannins. Indeed, in many studies, tannins showed toxicity to yeasts, filamentous fungi and bacteria; and the antibacterial effect of some medicinal plants extracts is explained by their content in tannins. It is also reported that tannins have an inhibitory effect on viral reverse transcriptases (Scalbert, 1991).

Coumarins, phenolic substances made of fused benzene and α-pyrone rings, are particularly known for their antithrombotic, anti-inflammatory and vasodilatatory activities. Warfarin is a well-known coumarin which is used both as an oral anticoagulant and, interestingly, as a rhodenticide; it may also have antiviral effects. Several other coumarins have shown antimicrobial activity against many fungi, gram-positive bacteria and viruses. An indirect negative effect on infections has been observed with some coumarins that stimulate macrophages.

The potential antimicrobial activity of **quinones**, aromatic rings with two ketone substitutions, oxidized phenols ubiquitous in nature and characteristically highly reactive,toward proteins has been discussed in Section 3.1.1. Anthraquinones, among which hypericin, isolated from *Hypericum perforatum*, are effective against many microorganisms (Cowan, 1999). Arbutin, a hydroquinone derivate, is effective against *Pseudomonas aeruginosa*. In *Pyrus ussuriensis*, arbutin is metabolized to hydroquinone, then to benzoquinone; this later compound is the substance essential for antibacterial activity of *Pyrus* spp.

3.2.2. Terpenoids

Many studies report the antibacterial, antifungal, antiviral and anti protozoal activities of terpenes and terpenoids, ubiquitous compounds synthesized from isoprene units and occuring as monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes according to their

numbers of carbons. A great part of essential oils, essentially composed of monoterpenoids, but also of sesquiterpenoids, phenols and/or C6-C3 units, are effective against fungi and bacteria; it is reported that 60% of essential oils derivatives examined are effective against fungi while 30% possess antibacterial effects. The triterpenoid betulinic acid has been reported to inhibit HIV (Cowan, 1999). The sesquiterpene artemisinin and its derivate α-artemether, find current use as antimalarials, the latter drug having been chosen by the scientific working group of the WHO as a treatment for cerebral malaria (Vishwakarma et al., 1992)..

3.2.3. Alkaloids

Alkaloids are a family of extremely diverse nitrogen compounds possessing many biological activities. The indoloquinoline alkaloids such as cryptolepine have a high antimycobacterial effect against *Mycobacterium tuberculosis* (MTB) and have recently been shown to be an alternative screening model to MTB for potential antitubercular drugs (Okunade et al., 2004). The bisbenzylisoquinoline (B.B.I.Q) alkaloids from Menispermaceae species have shown antimalarial, antifungal, antiviral, and antibacterial activities; for example cycleanin, a B.B.I.Q isolated from *Albertisia villosa*, has been found effective against bacteria, fungi and plasmodia (Otshudi et al., 2005). Berberine alkaloids are cationic antimicrobials produced by several *Berberis* medicinal plants, their antimicrobial effect is highly enhanced by flavonoids such as 5-MHC (Stermitz et al., 2000a).

3.2.4. Defensins

Plant defensins, which are also known as γ-thionins, are one of the most important and very well studied classes of antimicrobial peptides. They are produced by plants in order to defend themselves against invading microbial pathogens (Thevissen et al., 2007). Interestingly, plant defensins display antimicrobial activity, not only against plant pathogens but also against human microbial pathogens, including bacteria, fungi and parasites (Lay et Anderson, 2005).

The exact mechanism of the antimicrobial action of defensins remains a matter of controversy; there is nevertheless a consensus that these peptides selectively disrupt the cell membranes and the amphipathic structural arrangement of the peptides is believed to play an important role in this mechanism (Thomma et al., 2003). It is also reported that these antibacterial peptides, which are usually highly basic, recognize the acidic phospholipids exposed on the surface of the bacterial membrane (Thevissen et al., 2003). In the case of microbes, the anionic lipids are effectively present on the outer surface of the membrane whereas for mammalian cells, anionic lipids are present along the cytoplasmic side of the membrane. This feature might account for their preferential activity against bacteria but not against mammalian cells (Thevissen et al., 2004).

4. Effects of Plant Compounds on Antibiotic Resistance

Three main mechanisms of antibiotic resistance have been so far identified: *(i)* the ability of microorganisms to reduce the intracellular concentration of drug (reduced permeability, reduced uptake, active efflux); *(ii)* the inactivation of antibiotics; and *(iii)* the modification or elimination of the target site. Microorganisms may use one or more of these strategies to evade the inhibitory or lethal effects of a particular antibiotic and may transfer these capabilities to other strains, notably through plasmids.

The fight against antibiotics resistance implies on one hand the research for active compounds with a new mode of action, different of those described for existent antibiotics; that is the case of platensimycin, a previously unknown class of antibiotics produced by *Streptomyces platensis*. This antibiotic has a strong broad spectrum activity on Gram-positive strains by inhibiting the bacterial lipid biosynthesis, through the selective targeting of β-ketoacyl-(acyl-carrier-protein (ACP)) synthase I/II (FabF/B) (Wang et al., 2006).

A second approach in this fight against antibiotic resistance is the use of compounds without direct antimicrobial properties, but which enhance or restore the effects of antibiotics on resistant microorganisms (Shahverdi et al., 2004). This approach compares the effects of antibiotics by themselves to the combination of antibiotics and inactive compounds or plant extracts against resistant microorganisms. It is also possible to evaluate the effect of active plant extract (or compounds) in association, in sub-inhibitory concentrations, with antibiotics (Shahverdi et al., 2003). Different mechanisms of antibiotic resistance and their inhibitors from medicinal plants are summarized in Figure 1.

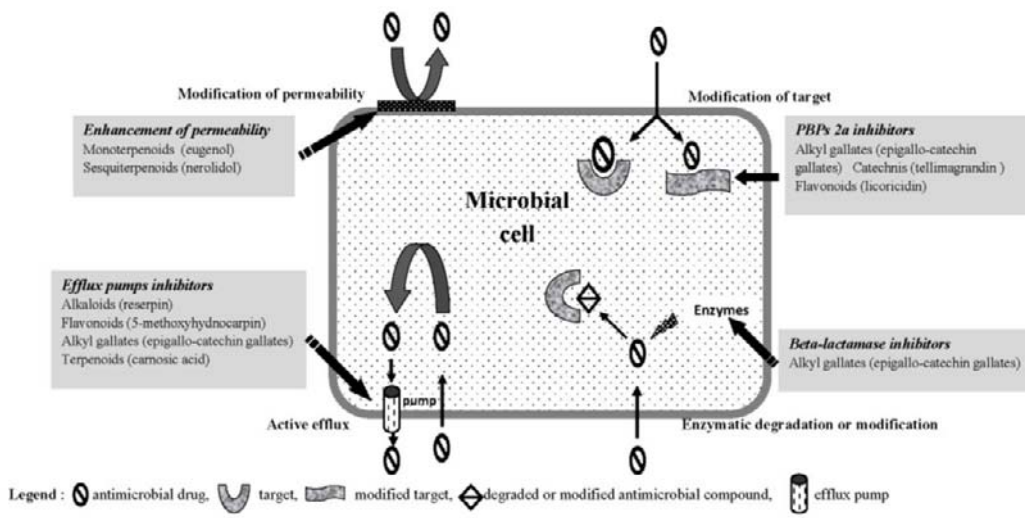

Figure 1. Mechanisms of antibiotic resistance and examples of their inhibitors from medicinal plants

4.1. Efflux Pumps Inhibitors

4.1.1. Efflux Systems

Among the major mechanisms involved in bacterial resistance, efflux pumps, responsible for the extrusion of the antibiotic outside the cell, have recently received a particular attention. These systems can confer resistance to a specific class of antibiotics or to a large number of drugs, thus conferring a multi-drug resistance to bacteria. Efflux pumps are currently classified into five families:

- ✓ ATP-binding cassette (ABC) transporters are ubiquitous membrane systems involved in different transport functions such as the efflux of toxins, metabolites and drugs. Bacterial ABC transporters involved in drug resistance are mainly drug-specific transporters and many of them are found in antibiotic producing organisms, such as *Streptomyces spp*, thus ensuring self-resistance to the drug they produce. In some Gram positive bacteria, such as *Staphylococci* and *Enterococci*, such specific transporters are also found, conferring resistance to macrolides and related compounds (Holland, 2003).
- ✓ Major facilitator superfamily (MFS) are ubiquitous systems ensuring transport of sugars, intermediate metabolites and drugs.
- ✓ Resistance nodulation and cell division (RDN) are involved in the transport of lipophilic and amphiphilic molecules or toxic divalent cations, also responsible for the solvent resistance of some bacterial strains. They are mainly found in Gram negative bacteria.
- ✓ Small multi-drugs resistance (SMR) are the smallest drug efflux proteins known, involved in the efflux of lipophilic cationic drugs.
- ✓ Multi-drug and toxic compounds extrusion (MATE) are mainly Na^+/drug antiporters in contrast to the other families of secondary transporters which act as proton/drug antiporters.

These five families of efflux pumps are commonly grouped in two major groups based upon bioenergetical and structural features: *(i)* the primary transporters which hydrolyze ATP as source of energy (ABC transporters); and *(ii)* the secondary transporters which use the proton (or sodium) gradient as source of energy (MFS, RDN, SMR and MATE). In bacteria, secondary transporters are dominant and many of them are multi-drugs resistance transporters, whereas ABC transporters are mainly specific drug resistance transporters.

In Gram positive bacteria, MDR is mainly conferred by MFS efflux systems, the most studied being NorA of *Staphylococcus aureus* and its homologues in *Bacillus subtilis*, Mmr and Blt. Whereas RDN efflux systems are major contributors to resistance for Gram negative bacteria, AcrAB-TolC and MexAB-OprM are also involved in the intrinsic resistance of *Escherichia coli* and *Pseudomonas aeruginosa*, respectively.

4.1.2. Efflux Pumps Inhibitors (EPIs)

The research of compounds inhibiting the efflux pumps is crucial in the fight against antibiotic resistance. These compounds are expected to: *(i)* decrease the intrinsic resistance of

bacteria to antibiotics; *(ii)* reverse acquired resistance; and *(iii)* reduce the frequency of resistant mutant strains emergence.

Medicinal plants extracts and natural compounds have been reported to inhibit efflux systems, thus enhancing or restoring the activity of diverse antibiotics. Among them:

4.1.2.1. Alkaloids

Reserpine, an antihypertensive alkaloid isolated from *Rauwolfia sp*, is known to inhibit the P-gp and potentiate the activity of fluoroquinolones on MDR Gram-positive bacteria; it enhances also the activity of tetracycline against MRSA strains and it has been shown to inhibit the LmrA of *L. lactis*, the ABC efflux system that confers MDR to this strain (Marquez, 2005). These effects of reserpine however appear at concentrations too high to be clinically relevant and moreover bacterial resistance to reserpine has been selected in some strains.

4.1.2.2. Flavonoids

Several *Berberis* medicinal plants producing berberine, an antimicrobial alkaloid, were found to also synthesize 5-methoxyhydnocarpin (5-MHC), an inhibitor of the NorA MDR pump of *S. aureus* (Stermitz et al., 2000a). 5-MHC, an amphipathic weak acid, distinctly different from the cationic substrates of the NorA, has no antimicrobial activity per itself but increases the intracellular accumulation of berberine and strongly potentiates its activity (Stermitz et al., 2000b).

4.1.2.3. Alkyl Gallates

Numerous biological properties have been reported for the green tea phenols, epigallocatechin gallates and epicatechin gallates, including antimicrobial activities, reversal of methicillin resistance or inhibition of P-gp. These compounds were also found to reverse tetracycline resistance in *Staphylococci* strains and to potentiate the activity of norfloxacin against a NorA over-expressing *S. aureus* (Hatano et al., 2005).

4.1.2.4. Terpenoids

Diterpens compounds, carnosic acid from *Rosmarinus officinalis* and isopimarane derivatives from *Lycopus europaeus*, potentiate the activity of erythromycin and tetracycline against a macrolide- highly resistant *S. aureus* harboring the ABC transporter MsrA (Brehm-Stecher and Johnson, 2003).

4.2. B-Lactamases Inhibitors

The production of β-lactamases, enzymes that hydrolyse the β-lactam ring of cephalosporins and penicillins, is the most determinant cause of resistance to β-lactams (Livermore, 1995).

To overcome β-lactamases related resistance, antibiotics association including non β-lactams can be used; an other approach, more interesting, is the use of β-lactams in association with inhibitors of β-lactamases. Clavulanic acid combination with amoxicillin demonstrates how successful this approach can be; this beta-lactamase inhibitor is combined

with penicillin group antibiotics to overcome resistance in bacteria which otherwise would inactivate most penicillins.

Some natural products, such as epigallo-catechin gallates inhibit the penicillinase produced by *S. aureus*, restoring the activity of penicillin (Zhao et al., 2002).

In efforts to find new β-lactamases inhibitors, sixteen Cameroonian medicinal plants were investigated. Seven extracts from *Mammea Africana, Garcinia lucida, Garcinia kola, Bridelia micrantha, Ochna afzelii, Prunus aficana and Adenia lobata*, presented interesting anti-β-lactamases activities (Zhao et al., 2002; Gangoue-Pieboji et al., 2007). These plant extracts are worthy of further investigation to isolate and identify the bioactive compounds, which can provide useful leads in the development of new β-lactamases inhibitors.

4.3. Inhibitors of PBP 2a

The production of penicillin-binding proteins (PBPs) 2a is the most important mechanism involved in the antibiotics resistance of methicillin-resistant *Staphylococcus aureus*. PBPs 2a interact with β-lactams, although much less strongly than other PBPs; modification of PBPs seems to be the favored mechanism of β-lactam resistance in gram positive bacteria, whereas β-lactamase production is favored in gram negative bacilli (Rice et al., 2003); the use of inhibitors of PBP 2a is an interesting approach to overcome resistant microorganisms.

Many studies report the enhancement or restoration of β-lactams activity against MRSA by natural compounds, including several catechins, notably the epigallocathechin gallate from green tea, tellimagrandin (tannin) and rugosin B from *Rosa canina*, corilagin (tannin) from *Arctostaphylos uva-ursi*, have been found to markedly reduce the MIC of β-lactams against MRSA, effect attributed to an inhibition of either PBPs 2a activity or its production (Gibbons et al., 2004; Hatano et al., 2005). The modulation of antibiotic resistance by inhibition of PBPs 2a is also encountered in flavonoids, such as licoricidin and licochalcone, which markedly reduce the MIC value of oxacillin against MRSA. It has been reported that licoricidin does not affect the production of PBP 2a in an MRSA strain, although it might still alter PBP 2a's inhibitory effect on cell wall production in some way.

4.4. Other Inhibitors of Antibiotic Resistance

As the reduction of bacterial membrane permeability to antibiotics is one of the mechanisms of resistance, restoring or enhancing the membrane permeability to antimicrobial drugs may be an attractive mean to overcome antibiotics resistance (Nikaido, 1994). The sesquiterpenoids nerolidol, farnesol, bisabolol, and apritone were investigated for their abilities to enhance bacterial permeability and susceptibility to antimicrobial compounds; flow cytometry studies suggest that enhanced permeability results from disruption of the cytoplasmic membrane (Brehm-Stecher et Johnson, 2003). In disk diffusion assays, treatment with low concentrations (0.5 to 2 mM) of these sesquiterpenoids enhanced the susceptibility of *Staphylococcus aureus* to ciprofloxacin, clindamycin, erythromycin, gentamicin,

tetracycline, and vancomycin. Nerolidol and farnesol also sensitized *Escherichia coli* to polymyxin B (Daugelavicius et al., 2000).

5. Conclusion

Antimicrobial medicinal plants play an important role in health care in traditional medicine and could yield compounds important for modern medical practice. In developing countries, where the impact of infectious diseases is particularly large, traditional healers predominantly recourse to medicinal plants. From these medicinal plants, most often used for millenia, many antimicrobial compounds have already been isolated and identified, supporting thus their traditional uses. In modern medical practice, medicinal plants have a major interest for their possible role in the fight against antibiotics bacterial resistance. Indeed, the alarming worldwide incidence of antibiotics resistance causes an increasing need for new compounds that can act either by a direct antimicrobial activity or by inhibiting resistance mechanisms of microorganisms of medical importance. Compounds belonging to several phytochemical groups and displaying an antimicrobial effect against bacteria, fungi, viruses and parasites have been isolated from medicinal plants, promising to enrich the arsenal of antimicrobial drugs, particularly against multi-resistant microorganisms. More interestingly, medicinal plants provide compounds inactive by themselves, but that can enhance or restore the activity of existing antibiotics. These compounds generally act by inhibiting resistance mechanisms of microorganisms to antibiotics, and may permit to extend the effective life span of currently used antibiotics or to recover antibiotics to which microorganisms have already developed resistance. The synergistic concomitant use of natural compounds and antibiotics can also help to reduce doses of the later in order to help avoiding adverse side effects.

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